GENETICS AND MECHANISMS OF TELOMERE-MEDIATED LUNG DISEASE

by
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Abstract

Chronic lung disease is the third leading cause of death in the United States. Emphysema and pulmonary fibrosis are two of the most common forms and are thought in part to represent a premature aging phenotype in the lung. This thesis aims to define the role of telomeres in the genetics of age-related lung disease. Using publically available sequencing data from patients with severe, early-onset COPD, we identified telomerase mutations as a novel Mendelian genetic cause of emphysema. The patients in this cohort showed a unique gene-environment interaction in which smoking was necessary for development of the emphysema phenotype, and female telomerase mutation carriers were particularly prone to developing emphysema. In a family with pulmonary fibrosis and idiopathic infertility we demonstrated a case of functional reversion in the proband, who carried both a germline dominant negative missense allele in the telomere binding protein *TINF2* and an acquired somatic deletion in *cis* that abolished expression of the missense allele in bone marrow-derived cells. In the final chapter, we identify mutations in the RNA biogenesis protein *NAF1* as a cause of autosomal dominant familial pulmonary fibrosis-emphysema. The mutations segregated with the disease phenotype and short telomeres and disrupted nuclear targeting of NAF1. Taken together, these genetic findings have immediate clinical implications in the lung transplant setting, as patients with short telomeres are especially sensitive to myelosuppresive regimens. They also point to telomere-mediated senescence as a key mechanism of age-related lung disease pathogenesis.

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Chapter 1:
The Short and Long Telomere Syndromes: Paired Paradigms for Molecular Medicine

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Summary

Recent advances have defined a role for abnormally short telomeres in a broad spectrum of genetic disorders. They include rare conditions such as dyskeratosis congenita as well pulmonary fibrosis and emphysema. Now, there is new evidence that some familial cancers, such as melanoma, are caused by mutations that lengthen telomeres. Here, we examine the significance of these short and long telomere length extremes for understanding the molecular basis of age-related disease and cancer.

The origins of telomere human genetics

Telomeres and telomerase were first discovered in *Tetrahymena thermophila*, a protozoan of no obvious clinical significance(1, 2). Genetic models in yeast, mice, and human cells subsequently established a framework for how chromosome ends are maintained before disease connections crystallized(3). Now, abnormally short telomeres are appreciated to mediate common age-related disorders such as pulmonary fibrosis and emphysema(4). While the clear connection between short telomeres and degenerative disease may have suggested a hypothetical benefit for long telomeres, new discoveries have uncovered a potential link between *long* telomeres and familial cancer syndromes(5-7). Here, we review how extreme telomere length abnormalities, both short and long, inform understanding the molecular basis of diseases associated with aging as well as cancer.

Telomere length is a "molecular clock" mechanism

Telomere shortening is considered one of the best-characterized mechanisms of cellular aging(4). This claim builds on the fact that telomere length predicts the onset of replicative senescence(8, 9). Telomeres also shorten in humans with age, and in the past decade, it has become clear that abnormally short telomeres recapitulate several premature aging phenotypes(4). The progressive shortening of the TTAGGG telomeric sequence occurs because DNA polymerases cannot fully copy to chromosome ends(10). Telomerase offsets this 'end replication problem' by synthesizing new telomere sequences(2, 11, 12). When telomeres become critically short, they activate a DNA

damage response(13), which provokes cellular senescence or apoptosis(14-18); these responses underlie the progressive disease phenotypes seen in disorders that share the short telomere defect as a driving mechanism.

Several safeguards restrict telomere elongation in favor of net shortening with aging. They include a tight regulation of telomerase levels, as well as intrinsic factors at telomeres that limit excessive elongation by telomerase(19-21). The expression of the reverse transcriptase component of telomerase, TERT, is also repressed in most adult tissues. In hematopoietic as well as other somatic stem cells, even though telomerase is expressed, its low levels do not offset the telomere shortening that normally occurs with aging(19, 22-24). As we will discuss here, genetic defects that disturb this telomere length homeostasis cause highly penetrant disease phenotypes.

The mammalian short telomere phenotype was first studied in telomerase null mice(14, 15, 25). While telomerase loss alone has no clinical consequences in the first generation, late-generation telomerase null mice accumulate short telomeres(14, 15, 18, 25, 26). The short telomeres cause degenerative organ failure indicating that the telomere length, and not telomerase loss, is the primary determinant of the phenotype. Late-generation mice with short telomeres develop a stem cell failure phenotype, which is prominent in highly proliferative tissues such as the bone marrow and intestinal tract where stem cell replicative potential is critical for homeostasis(14, 15, 18, 25, 26). The human short telomere syndromes recapitulate these phenotypes(21, 27).

THE SHORT TELOMERE SYNDROMES

The human short telomere phenotype in high turnover tissues

Studies over the past decade have linked the human short telomere phenotype to a broad spectrum of disease(21); it varies in severity and spans the entire age spectrum from infancy to adulthood (Figure 1). While at onset their clinical and histopathologic classification alone may show few shared features, a growing appreciation for their genetics has highlighted a unified natural history(28). Their recognition as a single syndromic spectrum is critical for treatment decisions, because even though a single organ presentation may arise initially, the systemic telomere defect complicates treatment. Because some of these complications can be averted, the molecular grouping of disease across organs under the short telomere syndrome umbrella exemplifies a molecular medicine paradigm that directly advances patient care(27, 29, 30).

The short telomere phenotype in children and young adults represents more severe disease(4). Bone marrow failure is its most common first manifestation, and stem cell transplantation alleviates this condition pointing to a stem cell-autonomous defect in this compartment(21, 31-34). Affected individuals are also prone to developing intestinal villous atrophy, immunodeficiency and infertility(21, 27, 35). Pediatric presentations may also be recognized in historically defined syndromic entities. Dyskeratosis congenita was the first disorder to be linked to telomerase mutations and short telomeres(36, 37); it is classically defined by abnormalities in the skin, mucosa and

nails(38, 39). Hoyeraal-Hreidarsson syndrome manifests in infancy and is characterized by developmental delay, enterocolitis, and immunodeficiency(27, 40-42). The criteria for recognizing Hoyeraal-Hreidarsson syndrome and dyskeratosis congenita may be specific; but they identify only a small subset of all short telomere syndrome presentations(43).

The slow turnover phenotype in short telomere syndromes

Lung disease is the most common presentation of short telomere syndromes and it represents an attenuated, adult-onset phenotype (Figure 1)(43). Two types of lung disease have been linked to mutant telomerase and telomere genes. Idiopathic pulmonary fibrosis and the related interstitial diseases are marked by progressive lung scarring. Familial pulmonary fibrosis is a common manifestation of short telomere syndromes(43), and mutant telomere genes explain one third of all cases(44-49). Telomerase mutations have also been recently linked to the risk of emphysema(50). The frequency of telomerase mutations in severe emphysema rivals alpha-1 antitrypsin deficiency, which until recently was its only known Mendelian cause(50). In families with telomerase mutations, emphysema appears in smokers, while pulmonary fibrosis is the predominant pathology in never smokers(50). This apparent phenotypic heterogeneity points to a profound gene-environment interaction in a Mendelian disorder in which a single mutation causing telomere shortening provokes fibrotic scarring in never smokers and emphysematous airspace destruction in smokers(50). In animal models, telomere dysfunction in alveolar stem cells triggers cellular senescence and recapitulates many features of the human lung pathology including recruitment of an inflammatory

response(51). The fibrosis-emphysema caused by short telomeres may therefore also result from stem cell failure, and this biology points to potential new approaches to their treatment(4, 51). Pulmonary fibrosis and emphysema are estimated to affect 100,000 and 5 million individuals, respectively, in the United States alone(52, 53). Their disease burden, along with their close association with telomere genetics, makes the short telomere syndromes the most prevalent among the premature aging disorders.

Cancer in the short telomere syndromes

Although the majority of the premature mortality in the short telomere syndromes is caused by degenerative organ failure, there is also an increased risk for cancer. Cancer is estimated to affect 10% of dyskeratosis congenita cases, and when it arises, it is diagnosed at a younger age than the general population(54). The basis of the cancer prone state in the short telomere syndromes is not understood, but its predilection appears to be for high turnover tissues where stem cell failure also occurs. Short telomere syndromes are associated with an increased incidence of non-melanoma skin cancers, as well as squamous cell carcinomas of the head and neck(54), but the highest risk is for myelodysplasia and acute myeloid leukemia. These latter bone marrow-derived malignancies are often a first manifestation of telomere-mediated disease(54, 55). In the bone marrow, the stem cell failure state and progressive stem cell dropout may cause replication errors in surviving stem cells that could lead to a clonal advantage. Short telomere patients may also have impaired cancer surveillance because of immunosenescence(21). All in all, even though the rate of some types of cancers in short

telomere syndromes is higher than in the general population, its overall incidence is dwarfed by organ failure which accounts for 90% of the mortality(38).

The genetics of short telomere syndromes

Short telomere syndromes show Mendelian inheritance, and as of this writing, 11 genes have been identified(34, 36, 48, 56-67) (Figure 2). Together, they explain 50-70% of the short telomere Mendelian phenotype. Mutations in these genes disturb telomere homeostasis by impairing telomerase biogenesis, affecting its catalytic functions, its recruitment by shelterin, or the stability of telomere replication machinery components (Figure 2). The predominant phenotype and age of onset do not depend on the gene or the mutation type, but is determined by the extent by which a given mutation causes telomere shortening(68). The most prevalent cause of short telomere syndromes is heterozygous loss-of-function mutations in *TERT*, which manifest in adults as autosomal dominant familial pulmonary fibrosis(4) (Figure 3A). In rare cases, biallelic *TERT* mutations have also been described and they are associated with more severe short telomere defects and early-onset disease(69). Mutations in *RTEL1* and *PARN* also show a similar pattern of adult- and pediatric-onset disease depending on whether one or two alleles are affected, respectively(48, 63, 70).

Genetic anticipation and the evolving disease pattern in the telomere syndromes

Two features distinguish the genetics of autosomal dominant short telomere syndromes from other Mendelian disorders; and they explain the natural history of these disorders.

The first is that autosomal dominant families with short telomere syndromes show genetic anticipation, an earlier and more severe onset of disease in each successive generations(57, 71) (Figure 3A). Telomere shortening is a second established molecular mechanism for genetic anticipation in addition to trinucleotide repeat expansion. The successive telomere shortening eventually precipitates severe pediatric disease and/or infertility, leading to loss of the mutation in that lineage(46, 68). Telomere gene mutations thus tend to be private to each autosomal dominant kindred, leading to significant allele heterogeneity. In addition to the earlier onset in later generations, the disease evolves from slow-turnover tissues in older generations (e.g. pulmonary fibrosis-emphysema) to a high-turnover phenotype in younger generations (e.g. bone marrow failure)(68). The rate of genetic anticipation and disease evolution within a family depends on the extent of telomere shortening caused by a given mutant allele across a generation(72). As we will highlight below, cancer prone families with mutations that may conversely promote telomere lengthening also show evidence of genetic anticipation.

THE LONG TELOMERE SYNDROMES

The price of long telomeres in an increased risk of melanoma

The premature aging phenotypes caused by abnormally short telomeres may intuitively suggest that long telomeres confer an advantage for health and lifespan. However, there is increasing evidence that such a view may be overly simplistic. In the past two years, mutations that appear to lengthen telomeres have been linked to an increased risk of cancer. It is these familial cancer syndromes that we posit here are *long* telomere

syndromes (Figures 1.1 and 1.3). They are also caused by mutations in telomerase and shelterin genes and two of these genes have been implicated also in the short telomere syndromes (Figure 1.2). The cancer spectrum in these putative long telomere syndromes is not yet fully defined, but so far it appears to be particularly enriched for melanoma and glioma(5-7, 73, 74). Notably, these cancers are different from the squamous cell and hematologic malignancies that arise in the short telomere syndromes.

Mutations in telomere genes that cause familial cancers

Evidence that mutant genes that may promote telomere lengthening cause familial cancer came first from a large melanoma kindred that carried an activating mutation in the *TERT* promoter(5). This mutation creates an E-twenty-six (ETS) binding site that turns on TERT transcription(5, 75, 76). Such a mechanism contrasts with the *TERT* loss-of-function mutations seen in the short telomere syndromes that impair the enzyme's catalytic functions(57) (Figure 3A). Somatic *TERT* promoter mutations are also found in 70% of melanomas(75) as well as a number of other solid tumors(77). Their high prevalence makes *TERT* promoter mutations some of the most common somatic mutations seen in human cancer and points to telomerase abundance as critical in cancer initiation and progression.

Since the initial description of a *TERT* promoter mutation in a family with melanoma, germline mutations in three other telomere gene, all encoding shelterin components, have been linked to familial melanoma and glioma(6, 7, 73, 74) (Figure 2). They include

POT1, *TPP1* and *RAP1*(6, 7) (Figure 2). Like *TERT*, mutations in *TPP1* can cause both short and long telomere syndromes. TPP1 is a shelterin component that has been implicated in telomerase recruitment, but it also functions to prevent telomerase access to the telomere(78-80). Its dual functions explain the contrasting net effect of TPP1 mutations on telomere length. The single *TPP1* mutation identified in short telomere syndromes is missing a single amino acid in the TEL patch domain; this impairs telomerase recruitment resulting in net shortening(61, 62). The resulting disease phenotype is marked by stem cell failure and a small increased cancer risk. In contrast, *TPP1* nonsense mutations in cancer prone families disrupt protein stability, and are predicted to allow improved telomerase access and promote telomere elongation(62, 73, 80). Based on the genetic anticipation documented for short telomere syndromes, it would be expected that long telomere length may similarly be expected to be inherited across generations(18, 26, 57, 81). A close examination of the published pedigrees interestingly shows a pattern of genetic anticipation for the age of melanoma-related diagnosis and mortality(6, 7, 73). While it is theoretically possible that lead time bias (i.e. the earlier detection of cancers because of earlier screening) could explain this pattern, the fact that this genetic anticipation occurs for the age at death as well as diagnosis favors a model where progressively longer telomeres promote and earlier onset and a more aggressive cancer course in later generations (Figure 3B).

How would abnormally long telomeres predispose to melanoma?

Studies over the past two decades in animal models shed light on how long telomeres

may promote cancer-related mortality. Although mice with short telomeres show limited survival because of their stem cell failure phenotypes, in cancer prone contexts they show a paradoxical advantage in overall survival(82-85). This benefit has been reproduced across cancer prone models, including oncogene-driven cancers, such as *Myc* and *K-ras,* as well as those driven by loss of tumor suppressors such as *Apc* and *Ink4a*(82-85). The survival advantage is seen despite the fact that short telomere mice accumulate more micro-tumors, because short telomeres induce the apoptosis and senescence checkpoints in these pre-cancers(83, 86). Thus, short telomeres confer an overall benefit at the organismal level in animal models where cancer is genetically induced by a single driving mutation.

The evidence from population studies also supports an association between long telomeres and the risk for some cancers and is best documented for malignant melanoma(87-90), an intriguing observation in light of the cutaneous melanoma-rich phenotype documented in the long telomere syndromes. Cutaneous malignant melanoma is marked by some of the highest mutation burdens among human cancers because of the mutagenic effects of ultraviolet light(91). Its clustering in families and individuals who have long telomeres underscores an important role for the telomere-mediated replicative senescence checkpoint in suppressing tumors where mutagenesis is environmentally induced. Unrestricted proliferation when telomeres are long would increase the likelihood of sustaining driver mutations that eventually promote a clonal advantage and metastasis. The role of long telomeres in promoting cancer is however complex and the

association between long telomeres and cancer risk may be tissue specific. For example, non-melanoma types of skin cancer, such as squamous cell carcinoma have been paradoxically associated with short telomeres(92). The available evidence thus suggests that distinct cancer phenotypes are associated with both short and long telomere syndrome extremes, and that a melanoma-rich phenotype will be a hallmark of long telomere syndromes.

SUMMARY AND LOOKING AHEAD

Emerging discoveries have painted a rich mosaic of how telomere length abnormalities at the extremes play a role in disease. The short telomere syndromes unite a group of stem cell failure disorders that share a single molecular pathology. Their grouping informs treatment and pathogenesis paradigms for common and poorly understood conditions especially lung disease. The short telomere phenotype overlaps with disease phenotypes that are normally acquired with aging, and it is associated with a modest cancer risk. At the other extreme, the long telomere syndromes manifest as a highly penetrant cancer predisposition. They are enriched for cutaneous melanoma presumably because in the setting of environmentally induced mutagenesis, the loss of the replicative senescence checkpoint promotes carcinogenesis. Both disease extremes point to the importance of a telomere length equilibrium in maintaining stem cell homeostasis with aging while simultaneously minimizing cancer risk.

The profound disease phenotypes caused by extreme telomere length disturbances raise

the possibility that targeting telomere length may be a plausible therapy strategy. In the short telomere syndromes, replenishing defective stem cells, such as is currently done with bone marrow transplantation, or aiming to elongate telomeres, could be clinically beneficial. In contrast, for long telomere-associated cancers, inhibiting telomerase could be effective in preventing or treating cancer. How much leeway is available to exploit this delicate system therapeutically remains to be determined, and any treatment approach will have to consider the multiple safeguards at telomeres that favor moderation over excess.

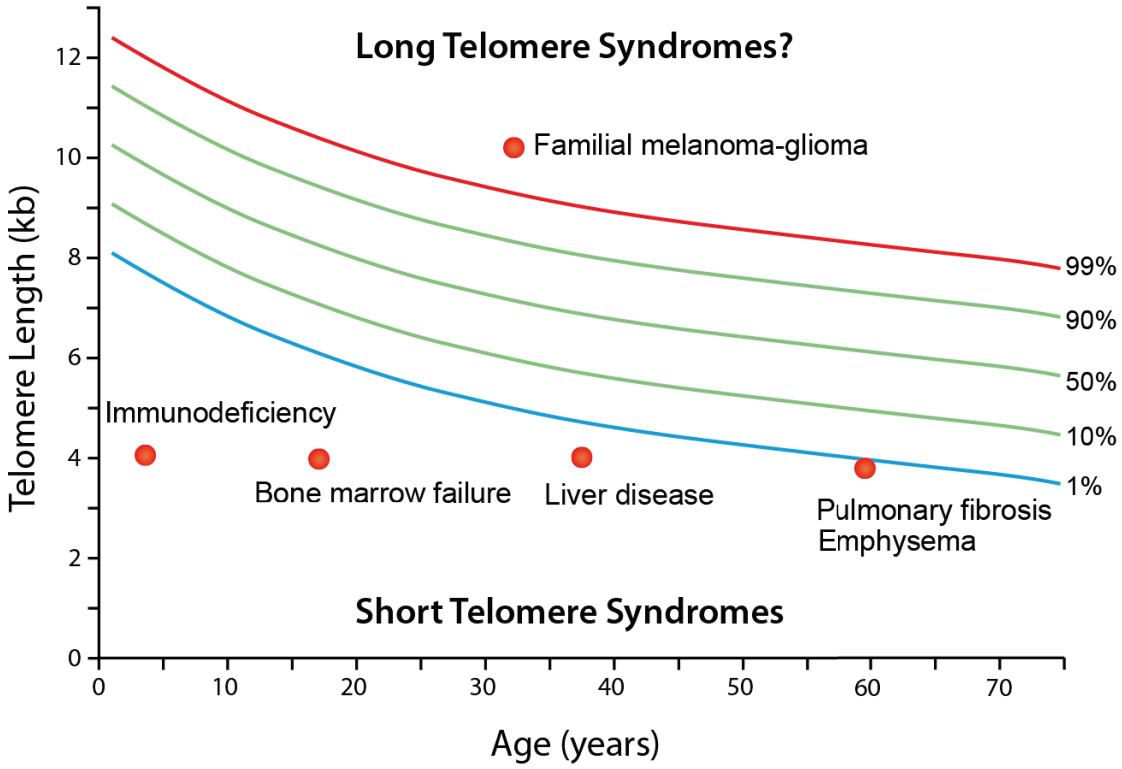

Figure 1. Telomere length extremes and their predominant clinical manifestations. Telogram showing the decreasing telomere length range across the age spectrum with percentile lines defining the normal range at every age. The short telomere syndromes have typical manifestations that are represented by the red circles at the typical age range of onset. Familial melanoma and glioma have been linked to mutations that putatively cause long telomeres. The typical presentations and age of onset for each of the presentation is also indicated.

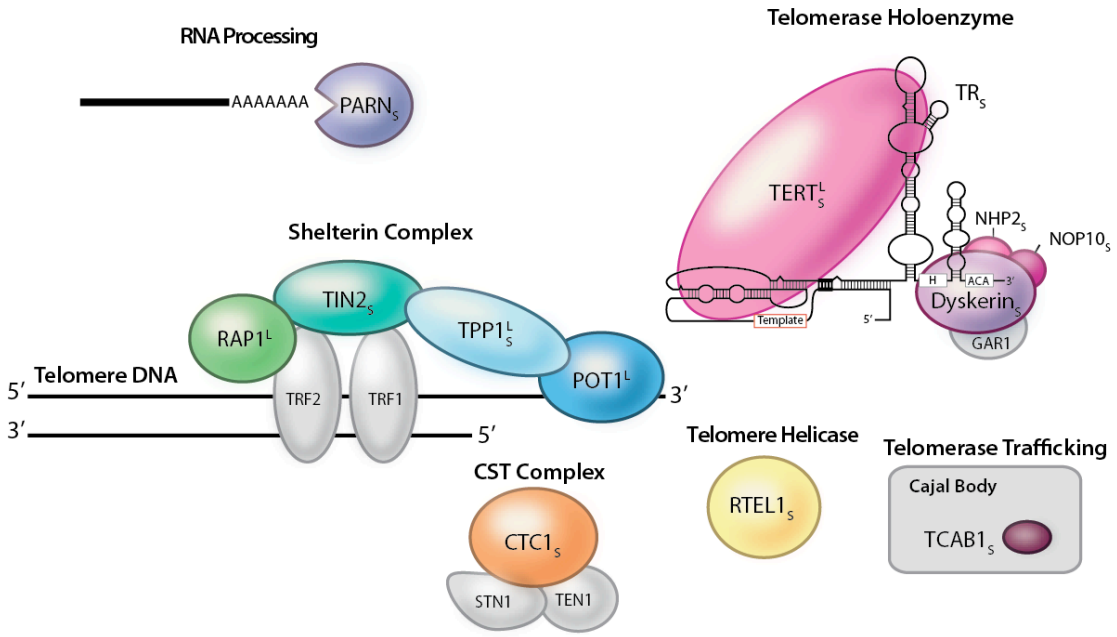

Figure 2. Telomerase and telomere components that cause short and long telomere syndromes. These mutations affect telomerase catalytic activity or processivity (TERT and TR), telomerase biogenesis (dyskerin encoded by *DKC1)*, NOP10 and NHP2, or its trafficking (TCAB1 also known as WRAP53). Mutations in short telomere syndromes also affect shelterin components TIN2 (encoded by *TINF2*), TPP1 (encoded by *ACD*), POT1 and RAP1 (encoded by *TERF2IP*). CTC1 and RTEL1 affect lagging strand synthesis and telomere replication, respectively. PARN is involved in RNA processing and deadenlyation; its function in telomere maintenance has not been fully characterized. The 'S' subscript indicates a link to short telomere syndromes (n=11 genes), while the 'L' superscript indicates a link to long telomere syndromes (n=4 genes). Mutant components are shown in color and gray denotes telomere components not linked to disease.

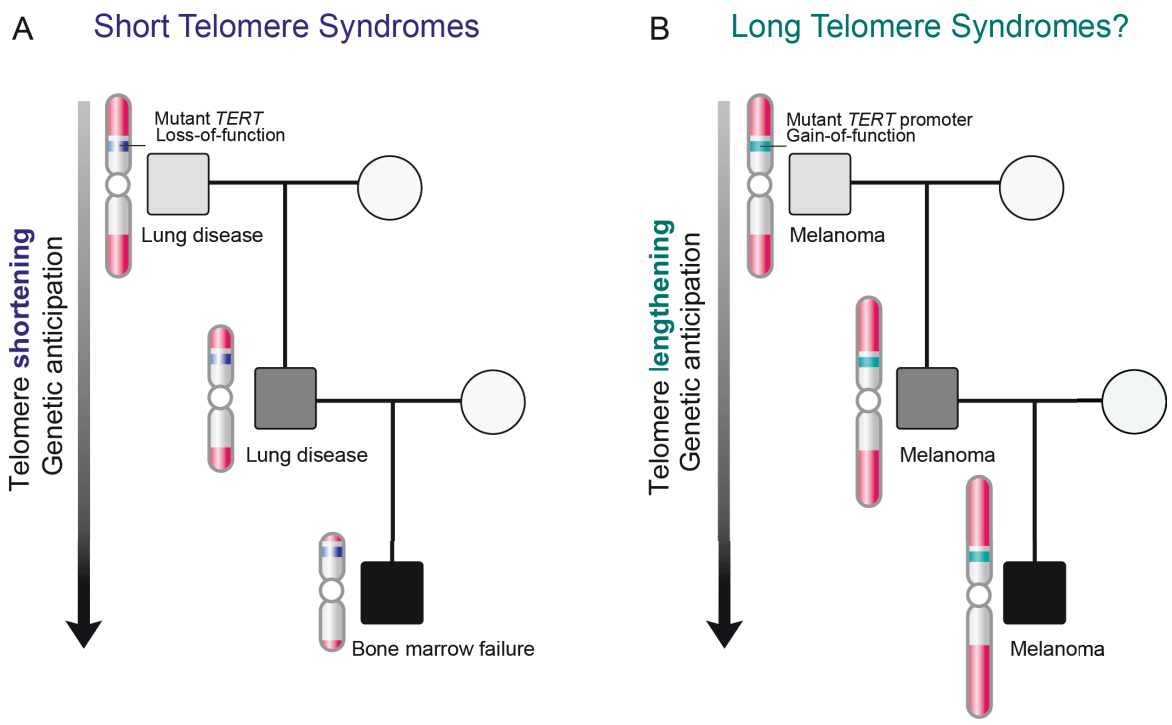

Figure 3. Genetic anticipation in the autosomal dominant short and long telomere syndromes. A. In the short telomere syndromes, successive telomere shortening across generations manifests in disease that shows an earlier age of onset. There is also an evolving pattern with ancestors developing lung disease and their progeny having a higher incidence of high turnover phenotypes such as bone marrow failure. **B.** Long telomere syndromes appear to also show genetic anticipation of the melanoma phenotype; this is caused by inheritance of long telomeres (right panel). Mutations in *TERT* have been linked to both syndromes with short telomere syndromes being caused by loss-of-function mutations (left), while familial melanoma is caused by promoter gain-of-function mutations that turn on TERT expression (right)**.**

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Chapter 2:
Telomerase mutations in smokers with severe emphysema

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Abstract

Mutations in the essential telomerase genes, *TERT* and *TR*, cause familial pulmonary fibrosis; however, in telomerase null mice, short telomeres predispose to emphysema after chronic cigarette smoke exposure. Here, we tested whether telomerase mutations are a risk factor for human emphysema by examining their frequency in smokers with chronic obstructive pulmonary disease (COPD). Across two independent cohorts, we found 3 of 292 severe COPD cases carried deleterious mutations in *TERT* (1%). This prevalence is comparable to the frequency of alpha-1 antitrypsin deficiency documented in this population. The *TERT* mutations significantly compromised telomerase catalytic activity and mutation carriers had short telomeres. Telomerase mutation carriers with emphysema were predominantly female, and had an increased incidence of pneumothorax. In families, emphysema showed an autosomal dominant inheritance pattern, along with pulmonary fibrosis and other telomere syndrome features, but manifested only in smokers. Our findings identify germline mutations in telomerase as a Mendelian risk factor for COPD susceptibility that clusters in autosomal dominant families with telomere-mediated disease including pulmonary fibrosis.

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death in the United States(1). Aside from cigarette smoke, age is its major risk factor. However, only a subset of smokers, approximately 10%, develops COPD, and the clustering of emphysema in families has suggested that genetic factors explain a significant portion of this susceptibility(2). Alpha-1 antitrypsin deficiency is the known Mendelian cause for emphysema(2). It manifests autosomal recessive inheritance because of biallelic mutations in the *SERPINA1* gene, and accounts young-onset, severe COPD in 0.5-1% of smokers of European descent(3). The other monogenic factors that underlie COPD susceptibility are not fully known(2, 4).

Telomeres are the DNA-protein structures that protect chromosome ends. Telomeres shorten with cell division and advancing age, and short dysfunctional telomeres signal a DNA damage response that provokes cellular senescence and apoptosis(5, 6). Telomerase is the specialized polymerase that synthesizes new telomere repeats(7-9). It has two core components: TERT, the telomerase reverse transcriptase, and TR, the telomerase RNA, that provides the template for telomere repeat addition(9, 10). Mutations in *TERT* and *TR* cause telomerase haploinsufficiency and the consequent short telomere defect is most frequently recognized in clinical settings as autosomal dominant pulmonary fibrosis(6). Even though lung disease is the primary life-threatening presentation in these patients, the telomere defect is systemic, and can manifest concurrently in a predictable, syndromic pattern that includes bone marrow failure, liver disease, and osteoporosis(11, 12). Because extrapulmonary telomere-mediated disease

can cause significant morbidity, recognizing this subset of pulmonary fibrosis patients has been shown to be relevant for treatment decisions in several settings(6, 12, 13).

We recently sought to understand the mechanisms by which telomere dysfunction causes pulmonary fibrosis by studying telomerase null mice with short telomeres. Although these mice have no obvious *de novo* lung defects, they surprisingly develop emphysema after chronic cigarette smoke exposure(14). Coincident with these findings, we identified a family with a deleterious *TR* mutation that included two female siblings who developed premature emphysema, and emphysema combined with fibrosis, after modest smoking histories(14). Another family was recently reported that had a similar clustering of emphysema with fibrosis in association with a *TERT* mutation(15). The observations in mice, in light of these anecdotal cases, led us to systematically test the hypothesis that mutations in telomerase may be a risk factor for emphysema in unselected populations. Using a candidate gene approach, in two cohorts of smokers with severe emphysema/COPD, along with pedigree data from a Johns Hopkins-based study, we show that deleterious telomerase mutations are a risk factor for COPD, and may occur at a frequency that is similar to alpha-1 antitrypsin deficiency in cases with severe, early onset disease.

Results

Rare *TERT* variants cluster with the COPD phenotype in two independent cohorts

To test whether telomerase mutations are a risk factor for emphysema, we examined the telomerase gene sequences in exome data from the COPDGene cohort, a study designed

to understand the genetic determinants of COPD susceptibility. These data have been recently made accessible through the National Heart Lung and Blood Institute (NHLBI) Exome Sequencing Project (Lung GO)(16). The clinical characteristics of the control group (n=206) and emphysema subjects (n=209) are summarized in Table 1. We designed a filtering strategy to identify novel or rare variants that could then be functionally examined (Supplementary Figure 1). In the control group, one rare *TERT* variant, Arg653Cys, was identified; however, this missense substitution did not affect telomerase catalytic activity (97 $\pm$ 5% of wild-type telomerase activity $\pm$ s.e.m, P=0.49, Student's *t*-test, Supplementary Figure 2). In contrast, among the smokers with emphysema, there were two heterozygous missense variants in *TERT*: Arg599Gln and Thr726Met (2 of 209, 1%) that we subsequently found to be functionally deleterious as shown below. The Arg599Gln variant was novel, and Thr726Met was previously reported in a child with bone marrow failure(17). We obtained archived DNA from the COPDGene parent study and confirmed the presence of these *TERT* variants by Sanger sequencing (Figure 1A).

We next tested the frequency of telomerase mutations in a second cohort of smokers with severe COPD. We queried cases from the Lung Health Study that we selected to match the age and forced expiratory volume in 1 second (FEV_1) criteria used in the COPDGene exome sequencing study, and examined the *TERT* and *TR* sequences along with the severe alpha-1 antitrypsin deficiency *SERPINA1* alleles (Table 1). Among 83 cases that fulfilled these prespecified criteria, we found no homozygous *SERPINA1* mutations, but we identified an individual with a heterozygous missense *TERT* mutation: His925Gln

(Figure 1A). This mutation was previously reported in several members of a family with pulmonary fibrosis and liver disease(18). In total, across the two COPD cohorts, there were three *TERT* variants among the severe COPD cases (3 of 292, 1%), and all of them fell in conserved motifs within the telomerase reverse transcriptase domain (Supplementary Figure 2.3). The *TERT* gene contains few deviations from reference sequence(19, 20), and none of these variants were found in 2,020 controls including 1,092 from the 1000 Genome Project, 528 published controls(20), and 400 individuals of similar European ancestry who we additionally sequenced (Supplementary Table 1). These data suggested a clustering of rare telomerase variants in smokers with severe emphysema.

TERT variants compromise telomerase function and telomere length

To test the functional significance of the rare variants identified, we reconstituted each of the TERTs in cells. We measured telomerase enzyme activity by quantifying the telomere products using the direct primer-extension assay. The three emphysema-associated *TERT* variants substantially compromised enzyme activity compared to wild-type telomerase as evidenced by the decreased intensity of the telomere repeat ladder ($P<0.001$, Student's *t*-test, Figure 1B-C). The reduction in activity was similar to pathogenic *TERT* and *TR* mutations documented in pulmonary fibrosis(12, 21). In contrast, the enzyme activity of the *TERT* variant from the control group was comparable to wild-type (Supplementary Figure 2). These data indicated that the clinical phenotype of severe emphysema enriched for individuals with rare, deleterious *TERT* mutations and that this clustering was statistically significant [3 of 292 COPD subjects (1%) vs. 0 of

2,226 controls (2,020 healthy controls and 206 control smokers), P=0.002, Fisher's exact test].

We examined the functional impact of the variants in vivo by measuring the telomere length. In two deceased subjects we measured telomere length using archived DNA by quantitative PCR, and found it was short relative to age-matched healthy controls (P=0.018, Student's t-test, Figure 1E), and comparable to *TERT* and *TR* mutation carriers with idiopathic pulmonary fibrosis (P=0.66). Telomere length in the telomerase-associated emphysema cases was also short compared to the control with the functionally intact *TERT* variant as well as the *SERPINA1* mutation carrier with alpha-1 antitrypsin deficiency in COPDGene (Figure 1E). We additionally measured lymphocyte telomere length by flow cytometry and fluorescence in situ hybridization (FISH) in the one living subject we could contact, and found it fell near the 10^{th} age-adjusted percentile, similar to mutation carriers with idiopathic pulmonary fibrosis that were also included in the quantitative PCR telomere length analysis (Figure 1C). These data supported the emphysema-associated *TERT* variants being functionally deleterious.

Telomerase mutations may be associated with a more severe emphysema phenotype

We examined the clinical data available from COPDGene and the Lung Health Study records and found all three mutation carriers were female with a mean age of 48 years at diagnosis (Supplementary Table 2). Chest CT scans in the COPDGene subjects showed apical airspace destruction, and one subject had additional interstitial lung abnormalities and bronchiectasis (Figure 2A-D). The dyspnea was severe requiring supplemental

oxygen support, and one subject died within five years of enrollment (Supplementary Table 2). Lung Health Study subjects had spirometry documented over five years, and, in the subject with the TERT His925Gln mutation, lung function declined faster than the lowest quartile of this 5,887 person cohort of smokers suggesting an accelerated disease course(22) (Figure 2E). Family history was not detailed in available study records, but we subsequently found the only living subject reported her mother, a smoker, was oxygen-dependent for the diagnosis of COPD.

Female gender is a risk factor for telomerase-associated emphysema

Mutations in telomerase and short telomeres are a risk factor for pulmonary fibrosis(19), and we sought to understand the predictors of emphysema onset compared to fibrosis. We examined the clinical histories of 50 telomere syndrome cases with lung disease that were consecutively recruited as part of a Johns Hopkins-based study (Table 2). Among never smokers, there were no cases of emphysema (0 of 39, 0%). However, among 11 smokers, we identified seven emphysema cases (64%). Notably, the emphysema cases were predominantly female with all the female smokers (6 of 6, 100%) developing either emphysema alone (n=2) or combined with fibrosis (n=4). In contrast, only 1 in 5 male smokers had radiographic evidence of emphysema (P=0.015, Fisher's exact test for enrichment of the emphysema phenotype in female smokers). These data suggested that short telomeres mediate a unique genetic-environmental interaction that predisposes to emphysema but only in smokers; this interaction seems to manifest most predominantly in females.

We analyzed the clinical features of telomere-associated emphysema phenotype by combining the Johns Hopkins cases with those we identified in the COPD cohorts (Table 3). In aggregate, 9 of the 10 cases were female. The average smoking history was 30 pack-years (range, 15-48), and of those that died, the mean age at the time of death from lung disease was 62 years (range, 46-68, n=7, Table 3). Notably, 3 of 9 subjects had spontaneous pneumothorax (33%), a rare, life-threatening complication of COPD that normally affects 5% of cases(23). Relative to historic estimates, the likelihood of recurrent spontaneous pneumothorax in this genetically uniform subset occurring by chance alone is low (P=0.016, Fisher's exact test). These clinical observations, albeit in a relatively small number, suggested that the telomere-associated COPD phenotype is associated with an increased risk for spontaneous air leaks.

Emphysema shows autosomal dominant inheritance with pulmonary fibrosis

We examined the pattern of inheritance in the five pedigrees of the Johns Hopkins emphysema cases. Four families carried known deleterious mutations in telomerase(12, 14, 21), including our originally reported family with the mutant *TR*, and one had classic features of a telomere syndrome(11). The emphysema phenotype in these pedigrees showed an autosomal dominant inheritance pattern with pulmonary fibrosis and other telomere phenotypes including bone marrow failure and liver disease (Figure 3A). The severity of the telomere defect did not predict the lung disease phenotype as both emphysema and fibrosis patients had equally abnormal short telomeres (Figure 3B). Notably, even within a single family that shared a telomerase mutation, emphysema appeared in female smokers showing an apical distribution, while female non-smokers

developed pulmonary fibrosis (Figure 3A-3C). These data indicated that telomere-mediated emphysema manifests as autosomal dominant trait, along with pulmonary fibrosis, but only appears in smokers.

Discussion

We report here germline mutations in telomerase are a risk factor for severe emphysema in smokers. Because telomere dysfunction lowers the threshold to emphysema in animal models, we tested whether telomerase mutations predispose to human emphysema, and found, in two independent cohorts, 1% of cases carried deleterious mutations in *TERT*. This frequency, although it constitutes a relatively small subset, is similar to that reported for alpha-1 antitrypsin deficiency in matched COPD populations(3). The emphysema-associated *TERT* variants compromised telomerase catalytic activity, and mutation carriers had abnormally short telomeres. Although a family history for pulmonary fibrosis was not detailed in the COPD cohorts we studied, in the families we fully characterized, emphysema showed autosomal dominant inheritance with pulmonary fibrosis and other telomere phenotypes. The familial clustering of emphysema with fibrosis suggests that these two lung phenotypes, heretofore considered distinct pathologies, may in some cases represent a continuum of degenerative lung disease that shares telomere dysfunction as a genetic susceptibility. For emphysema in contrast to fibrosis, the cigarette smoke exposure is a necessary second hit.

The evidence we document in human emphysema is compelling because short telomeres are a determinant of emphysema susceptibility in telomerase null mice(14). In these

animals, short telomeres lower the threshold to damage caused by cigarette smoke in epithelial cells(14). The additive damage of these 'two hits' provokes a DNA damage response that causes epithelial senescence(6, 14). Senescence and the resultant loss of regenerative capacity may thus be critical events that drive the airspace destruction in telomere-mediated emphysema (Alder and Armanios, unpublished). Telomere length is normal in emphysema patients with alpha-1 antitrypsin deficiency(24). Our data, in light of these observations, indicate telomere dysfunction may be a second, independent mechanism of emphysema susceptibility that is distinct from protease imbalance delineated in alpha-1 antitrypsin deficiency.

Several pieces of evidence suggest that short telomeres may play a broader role in emphysema susceptibility beyond the small subset of cases we identified. First, the coverage for *TERT* in COPDGene included only 75% of the coding sequence similar to what has been seen previously(25). Moreover, in addition to *TERT* and *TR*, a number of other telomerase and telomere genes have been implicated in the monogenic telomere disorders(26, 27). It is therefore possible that although the telomerase genes may account for a small subset, other mutant telomere pathway genes will collectively explain a larger proportion of susceptibility. Even when telomerase is wild-type, telomere length is genetically determined and short telomeres are is sufficient to predispose to degenerative disease in the lung and elsewhere(28, 29). The prevalence of telomerase and telomere gene mutations in smokers with emphysema will require future confirmation in other cohorts.

Our findings are significant for patient care because individuals with telomerase mutations are at risk for recurrent syndromic features including liver disease, osteoporosis and certain malignancies(6). Some of these same telomere syndrome morbidities are known to occur at higher frequency in patients with severe emphysema(30). Our data suggest the inherited telomere defect may play a role in simultaneously predisposing to these systemic comorbidities along with the lung disease. A telomere-mediated sub-phenotype of COPD may thus require individualized clinical care algorithms. Identifying telomere syndrome patients at the bedside is particularly important in the setting of lung transplant since some of these patients may be at increased risk for serious toxicities of immunosuppressive medications because of limited reserves in the bone marrow, gastrointestinal tract and elsewhere(13). Relatives of telomerase mutation carriers may also be at risk for telomere syndrome complications which may occur at an earlier age in successive generations because of genetic anticipation(12). Given the public health burden of COPD, our report suggests that emphysema may be a recurrent manifestation of telomere syndromes in populations where smoking remains prevalent.

Methods

Subjects

COPDGene Study. We accessed the Database of Genotypes and Phenotypes (dbGaP) on March 1, 2013 after approval from the Johns Hopkins Medicine Institutional Review Board and the National Heart Lung and Blood Institute (NHLBI) Data Access Committee. We analyzed the dbGaP clinical and exome data for the COPDGene study(31), and examined the *TERT*, *TR* and the *SERPINA1* gene sequence (phs000179.v3.p2 and pht002239.v2.p2.c1). Supplementary Figure 2 summarizes the depth of coverage for the coding and exon-flanking sequences of the candidate genes.

The COPDGene subjects who underwent exome sequencing (total n=415) were smokers who were selected for extreme phenotypes(32). Case subjects were selected to enrich for a severe, early-onset phenotype: younger than 63 years, with severe or very severe obstruction [forced expiratory volume in 1 second (FEV_1) less than 50% of predicted values], and had greater than 15% emphysema on CT scan (n=209)(32). Controls were older than 65 years, had FEV_1 greater than 80%, and less than 5% emphysema on CT (n=206). Quantitative chest CT scan assessment was based on the percentage of the lung with low attenuation areas below -950 Hounsfield units. Although subjects with known severe alpha-1 antitrypsin deficiency were excluded from COPDGene based on protein phenotyping, one PI*ZZ subject was inadvertently included. To verify exome variant calls, we obtained and sequenced archived DNA from the COPDGene investigators after ancillary study approval.

Lung Health Study. To test the hypothesis in a second cohort, we selected Lung Health Study subjects who fulfilled similar criteria to COPDGene for candidate gene sequencing. Smokers had FEV_1<50% of predicted values and were younger than 65 years (n=83). Lung Health Study participants had mild or moderate obstruction at the time of study entry, so we used selection criteria to identify subjects who developed severe obstruction at five years, the last timepoint pulmonary function was documented.

Johns Hopkins Registry. Families were recruited through the Johns Hopkins Telomere Syndrome Registry from July 1, 2005 to June 30, 2014. The study aims at understanding the genetics and natural history of telomere-mediated disease(33). The study was approved by the Johns Hopkins Medicine Institutional Review Board, and all the subjects gave written informed consent. Lung disease type was assessed for each of the subjects by CT imaging and review of the medical records including pulmonary function studies and death certificates. Chest CT images were available for review in 90% of the subjects, and, in the remaining cases, chest X-Ray and death certificate information were ascertained to determine the diagnosis.

Exome sequence analysis. Sequence files were annotated using publicly available software(34). Variants were selected for functional analysis if they had been absent in 1000 Genomes(35) as well as dbSNP build 130 (http://www.ncbi.nlm.nih.gov/SNP/), an uncontaminated earlier version of variants, and additionally had less than 0.001 minor allele frequency in the Exome Variant Server Database

(http://evs.gs.washington.edu/EVS). Candidate variants that fulfilled our filtering criteria from the exome data were confirmed by Sanger sequencing as previously described(21). We also manually sequenced *TR* in COPDGene subjects by PCR because of low coverage(21) (Supplementary Figure 4). The a priori designed filtering strategy is summarized in Supplementary Figure 1.

Targeted sequencing. To screen for telomerase mutations in Lung Health Study subjects, we designed and validated a TruSeq Custom Amplicon probe set (Illumina) that included the coding and flanking sequences of *TERT*, *TR* as well as exon 6 of *SERPINA1* containing the PI*Z allele that is mutated in more than 90% of alpha-1 antitrypsin deficiency cases(3). Libraries were generated from 250 ng DNA and analyzed on a MiSeq sequencer (Illumina). Of 83 samples sequenced, 7 samples (8%) had suboptimal coverage (less than 50% at 8X depth); the coverage for the 76 samples that passed quality control is summarized in Supplementary Figure 3. Supplementary Table 3 lists common *TERT* variants found in the COPDGene and Lung Health Study subjects.

Telomere length measurement. Telomere length was measured on peripheral blood lymphocytes by flow cytometry and FISH as previously outlined(21). In deceased subjects, telomere length was measured using archived DNA by quantitative PCR(36). For these studies, control and COPDGene genomic DNA was extracted from whole blood using the Gentra Puregene method (Qiagen). Each run included three replicates, and the mean from three independent runs was calculated.

Telomerase activity assay. The functional consequences of all the rare variants from the COPDGene and Lung Health Studies were examined using the direct telomerase activity assay. Wild-type and variant telomerases were reconstituted in vivo using 293FT cells (Invitrogen, Carlsbad, CA) by transient transfection with pcDNA-3xFLAG-hTERT and pBS-U1-hTR(37). The reconstituted telomerase was then immuno-purified from cell lysates and analyzed by the direct primer-extension assay at physiologic nucleotide concentrations as previously validated and described(29, 37). The 10 μL direct primer-extension reaction contained 5 μM dTTP, 5 μM dATP, 5 μM dGTP, 0.165 μM α-^{32}P-dGTP (3000 Ci/mmol, 10 mCi/mL, Perkin Elmer) and 1 μM $(TTAGGG)_3$ DNA primer in 1X telomerase reaction buffer (50 mM Tris-HCl pH 8.3, 2 mM DTT, 0.5 mM $MgCl_2$ and 1 mM spermidine). Comparable wild-type and mutant telomerase expression in transfected 293FT cells (Invitrogen) was confirmed by western blot for the FLAG-tagged TERT protein (anti-FLAG, clone M2, Sigma-Aldrich) and GAPDH (clone 6C5, Ambion) as an internal control(38). Comparable immuno-purification efficiency of telomerase was also confirmed by northern blot of TR extracted from immuno-purified telomerase(39). Telomerase activity was determined by measuring the total intensity of telomerase-generated products on the gel and normalizing against the internal loading control (^{32}P end-labeled 18-mer oligonucleotide) and the TR level measured by northern blot from immuno-purified telomerase(39). Quantification was based on four activity assays using cell lysates from two independent transfections.

Statistics

We used GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. A two-sided P-value of <0.05 was considered statistically significant, and all the P-values shown are two-sided.

Table 1. Clinical characteristics of subjects analyzed for telomerase gene sequences

	COPDGene		Lung Health Study
Subjects	**Controls (n=206)**	**Cases (n=209)**	**(n=83)**
Age at FEV_1 assessment (y)	**70.4 (65-81)**	**57.4 (46-63)**	**56.8 (41-64)**
Gender			
Male	**92**	**109**	**45**
Female	**114**	**100**	**38**
Ethnicity (self-reported)	**European American**	**European American**	**European American**
Smoking history (pack-years)	**50.0 (25-120)**	**50.1 (10-117)**	**47.4 (10-129)**
Smoking status*			
Current	**41**	**50**	**72**
Former	**165**	**159**	**11**
FEV_1 Baseline (L/s)	**2.60 (1.41-4.27)**	**0.93 (0.30-1.98)**	**2.10 (1.20-3.06)**
FEV_1 5-year visit (L/s)	**-**	**-**	**1.38 (0.69-2.01)**
GOLD Stage*			
0	**206**	**0**	**0**
1	**0**	**0**	**0**
2	**0**	**0**	**0**
3	**0**	**104**	**82**
4	**0**	**105**	**1**

FEV_1, Forced expiratory volume in 1 second; GOLD, Global Initiative for Chronic Obstructive Lung Disease (a scale used to measure chronic obstructive lung disease severity)

*Smoking status and GOLD stage are from year 5 data for the Lung Health Study subjects

Table 2. Characteristics of consecutive telomere syndrome cases with parenchymal lung disease (n=50)*

	Male	Female
Number of subjects	**25**	**25**
Age at diagnosis (y)	**53.1 (32-77)**	**54.2 (34-68)**
Smoking status		
Never smoker	**20**	**19**
Smokers	**5**	**6**
Lung disease relative to smoking history		
Smokers with emphysema	**1**	**6****
Smokers with fibrosis	**4**	**0**
Never smokers with emphysema	**0**	**0**
Never smokers with pulmonary fibrosis	**20**	**19**
Smoking history (pack-years)	**24 (10-41)**	**30 (25-37)**

*Subjects are included from 31 families (*TERT*, n=15; *TR* n=6, other telomere gene mutation n=5, clinical telomere syndrome or dyskeratosis congenita n=5)

**P=0.015 for proportion of female smokers with emphysematous changes relative to males (Fisher's exact test, two-sided).

Table 3. Clinical characteristics of telomerase mutation carriers with emphysema alone or combined with fibrosis (n=10)

Age at Diagnosis (y)	Gender	Smoking History (pack-years)	Genetic Diagnosis	Interstitial Lung Disease	Pneumothorax	Reference
34 *(d.46)*	F	15	*TR* del375-377	Severe UIP	Yes	Family 2
44	F	29	*TR* del375-377	None	Yes	Family 2
45	F	18	*TERT* His925Gln	Imaging not collected	History not collected	Lung Health Study
48 *(d.49)*	F	32	*TERT* IVS9-2 A→C	Severe	No	Family 4
44 *(d.54)*	F	25	Dyskeratosis Congenita	Minimal UIP	No	Family 5
49 *(d.62)*	F	43	*TERT* Arg599Gln	None	Yes	COPDGene
53	F	48	*TERT* Thr726Met	None	No	COPDGene
60 *(d.67)*	F	30	Dyskeratosis Congenita	None	No	Family 5
62 *(d.63)*	F	37	TR 98G→A	Moderate	No	Family 1
63 *(d.66)*	M	20	*TERT* Val747fsX20	Moderate UIP	No	Family 3

d., refers at age at the time of death from lung disease; UIP, usual interstitial pneumonia, the hallmark of idiopathic pulmonary fibrosis

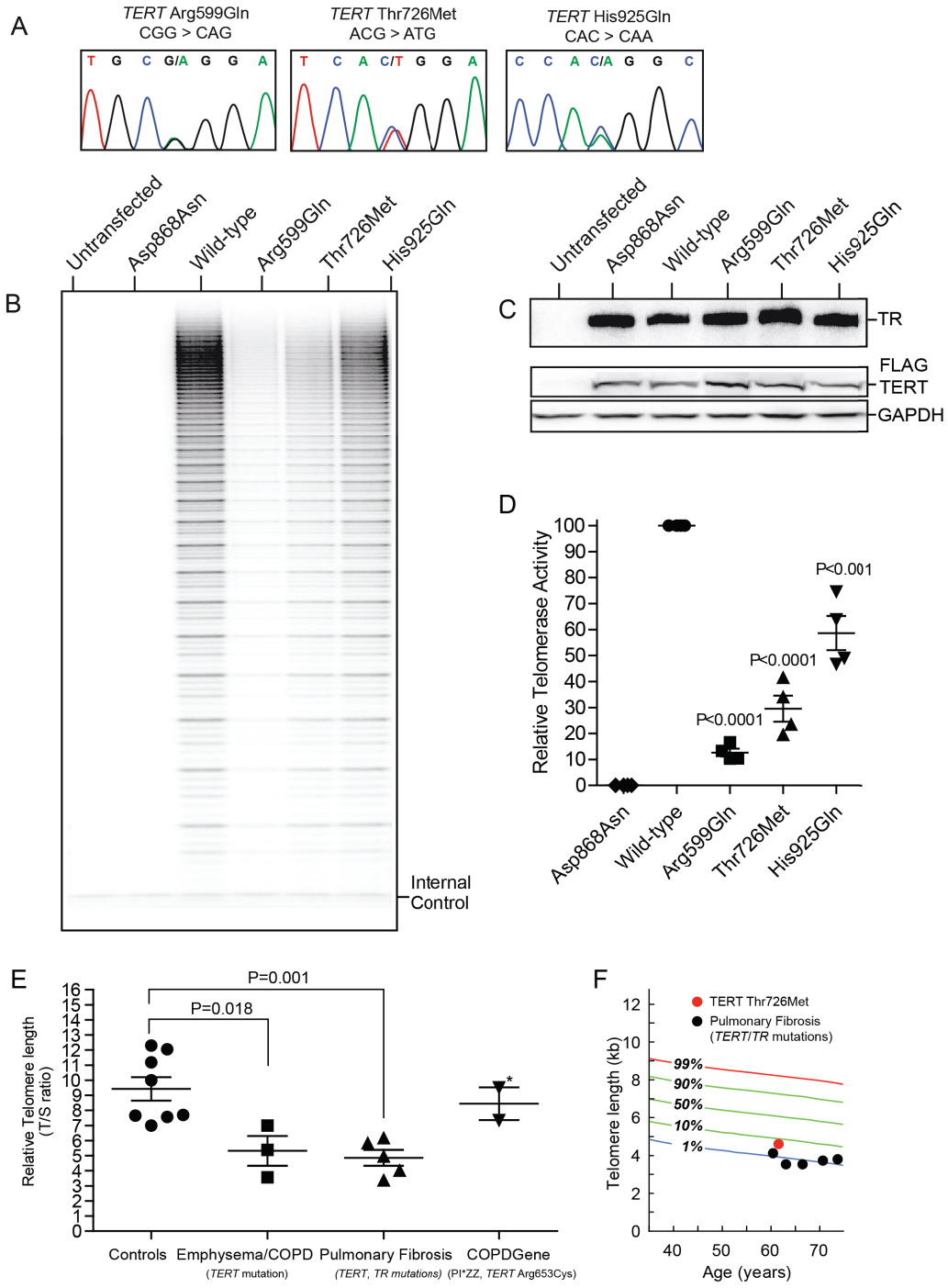

Figure 1. Functional consequences of telomerase variants identified by chronic obstructive pulmonary disease subjects. **A.** Chromatograms of PCR-amplified variants identified by next-generation sequencing. **B.** Gel image of telomere repeat ladder generated from wild-type and mutant telomerases reconstituted in vivo and immunopurified. The decreased intensity of the DNA repeat products generated by variant telomerases reflects impaired enzymatic activity of TERT Arg599Gln, Thr726Met and His925Gln. TERT Asp868Asn is a negative control, catalytically defective in one of the aspartic acid residues essential for reverse transcription. ^{32}P end-labeled 18mer oligonucleotide was included as an internal control for the recovery of DNA products. **C.** Northern blot for TR levels from immuno-purified telomerases (top). Western blot

for TERT expression in cells (bottom) was performed with anti-FLAG and anti-GAPDH antibodies for ectopically expressed FLAG-tagged TERT and endogenous GAPDH, respectively. **D.** Mean telomerase activity was derived from four activity assays from cell lysates prepared from two separate transfections. **E.** Relative telomere length as measured by quantitative PCR in age-matched controls: (ages 37-64, n=8), *TERT* mutation carriers (ages 46-57, n=3) from COPDGene and the Lung Health Study (LHS), telomerase mutation carriers with pulmonary fibrosis (ages 45-63, *TERT* n=2, *TR* n=3), and COPDGene controls: a homozygous *SERPINA1* Glu366Lys mutation carrier (formerly coded Glu342Lys, rs28929474, PI*ZZ genotype, age 46) and the control TERT Arg653Cys variant (age 68). **F.** Lymphocyte telomere length by flow cytometry and fluorescence in situ hybridization of a *TERT* mutation carrier and telomerase mutation carriers with pulmonary fibrosis relative to a nomogram of 400 controls. Error bars represent standard error of the mean, and two-sided P-values were calculated using Student's *t*-test.

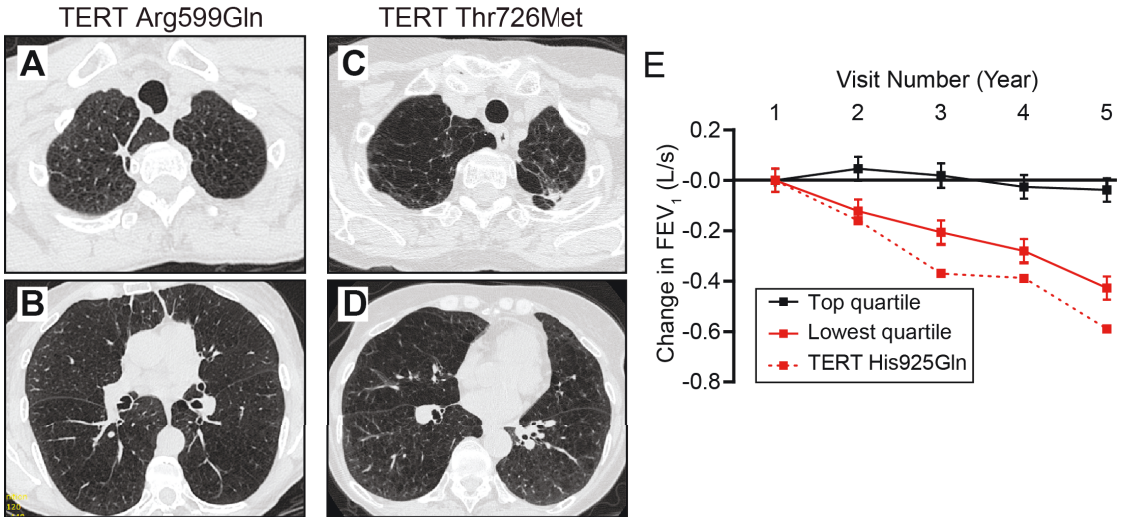

Figure 2. Radiographic and pulmonary function studies of telomerase mutation carriers with chronic obstructive pulmonary disease. A-D. High resolution inspiratory computed tomography (CT) images from COPDGene study subjects with telomerase mutations. The panels are each labeled at the top with the subject's mutation. Images from *TERT* mutation carriers show apical centrilobular emphysema **(A-B** and **C-D).** In the subject with TERT 599Gln, bronchiectasis and a reticular, subpleural interstitial lung abnormality could also be appreciated **(B)**. **E.** Rate of change in FEV_1 from baseline across the five years of the Lung Health Study in the subject with *TERT* His925Gln mutation. The rate of change is graphed relative to the highest and lowest quartiles of the 5,887 study population (+/- standard error of the mean).

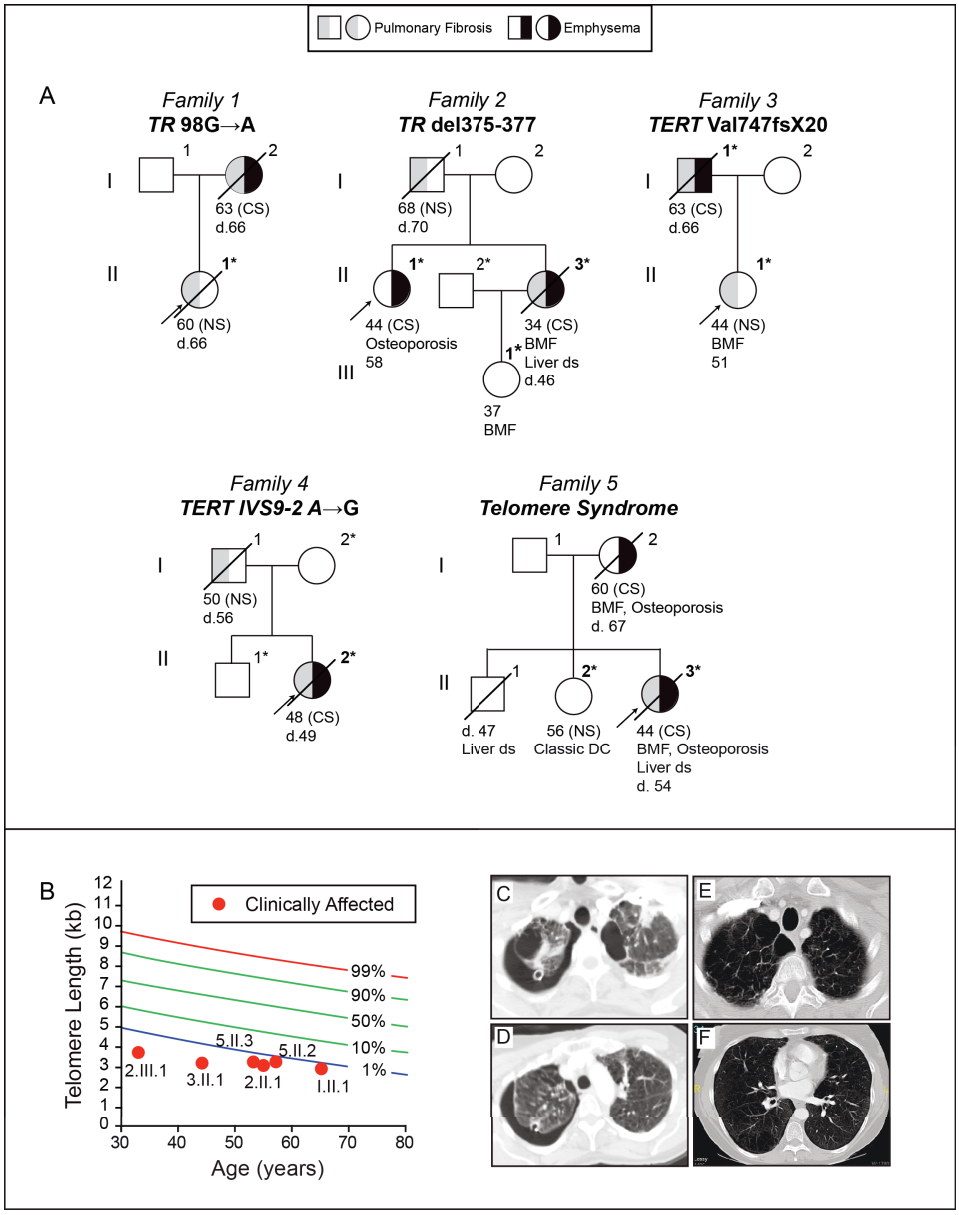

Figure 3. Pedigrees of telomere syndrome cases with emphysema. A. Pedigrees of emphysema cases with telomere defects and their relatives' clinical history. DC refers to dyskeratosis congenita, a telomere syndrome defined by mucocutaneous features. The * denotes individuals with DNA sequence data available and/or telomere length measurement performed. The bolded identifiers refer to individuals who carried the mutant gene and/or had very short telomeres (shown in **B**). CS refers to a positive smoking history; NS refers to never smokers, and BMF refers to bone marrow failure. The age at death from lung disease is indicated as "d." **B.** Lymphocyte telomere length by flow cytometry and fluorescence in situ hybridization shows the short telomere defect in affected members relative to age-matched controls. The nomogram was based on data from 400 controls. **C&D and E&F.** Apical and mid-lung chest CT cuts from two female cases (2.II.1 and 5.II.3, respectively) show severe apical emphysema with blebs. In addition, 2.II.1 has a right-sided pneumothorax that arose spontaneously.

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Supplementary Table 1. Allele frequencies of rare *TERT* variants

Variant	1000 Genomes (n=1,092)	Mutli-ethnic Cohort (n=528)*	Other Controls (n=400)**	Exome Variant Server (n=6,503)	LHS Subjects (n=83)	Minor Allele Frequency
TERT **Arg599Gln CGG>CAG**	**0**	**0**	**0**	**1**	**0**	**5.8×10^{-5}**
TERT **Arg653Cys CGT>TGT†**	**0**	**0**	**0**	**1†**	**0**	**5.8×10^{-5}**
TERT **Thr726Met ACG>ATG**	**0**	**0**	**0**	**4††**	**0**	**2.3×10^{-4}**
TERT **His925Gln CAC>CAA**	**0**	**0**	**0**	**0**	**1**	**5.8×10^{-5}**

LHS, Lung Health Study; MAF, minor allele frequency

**TERT* sequence data from multi-ethnic cohort examined in Yamaguchi et al. *NEJM* 2005.
***DNA from these controls of European descent was obtained through the National Disease Research Interchan
†This variant was present in the COPDGene control group had comparable activity to wild-type telomerase
††This variant was reported previously in a child with aplastic anemia in Liang J et al. *Haematologica* 2006.

Age at Diagnosis (y)	Age at Enrollment (y)	Gender	Mutation	Parent Study	Smoking History (pack-years)	BMI (kg/m2)	Pulmonary Function Tests			CT Findings	Resting saturation (%) (Hours on oxygen)	Co-morbidities
							FEV_1 (L/s, %)	FVC (L, %)	FEV_1/FVC (%, %)			
46	41	F	*TERT* His925Gln CAC>CAA	Lung Health Study	18*	21.3	1.34 (47)	2.79 (79)	48 (58)	-	-	-
49	57 *(d. 62)*	F	*TERT* Arg599Gln CGG>CAG	COPDGene	43	19.4	0.45 (20)	1.65 (56)	27 (35)	Emphysema (30%) Interstitial abnormality Bronchiectasis Expiratory air trapping	82 (24 h)	Pneumothorax
53	57	F	*TERT* Thr726Met ACG>ATG	COPDGene	48	18.9	0.69 (26)	2.16 (63)	31 (41)	Emphysema (40%)	95 (6 h)	Stomach ulcers Vertebral fractures Osteoporosis Congestive Heart Failure

Supplementary Table 2. Clinical features of subjects with *TERT* mutations in the COPDGene and Lung Health Studies.

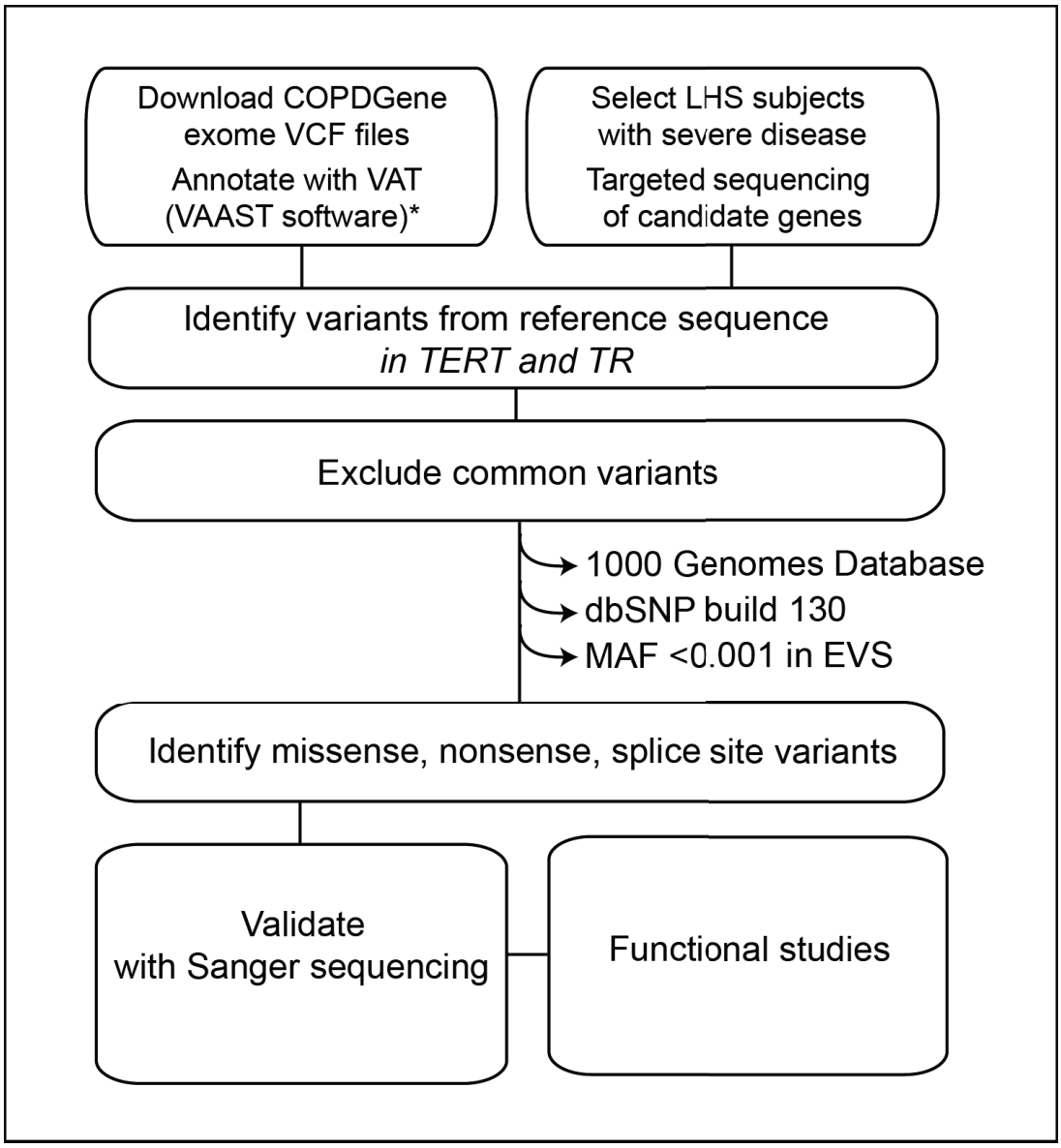
Abbreviations: BMI, body mass index; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity

*Smoking history was recorded at study enrollment; pulmonary functions are from year 5 of study.

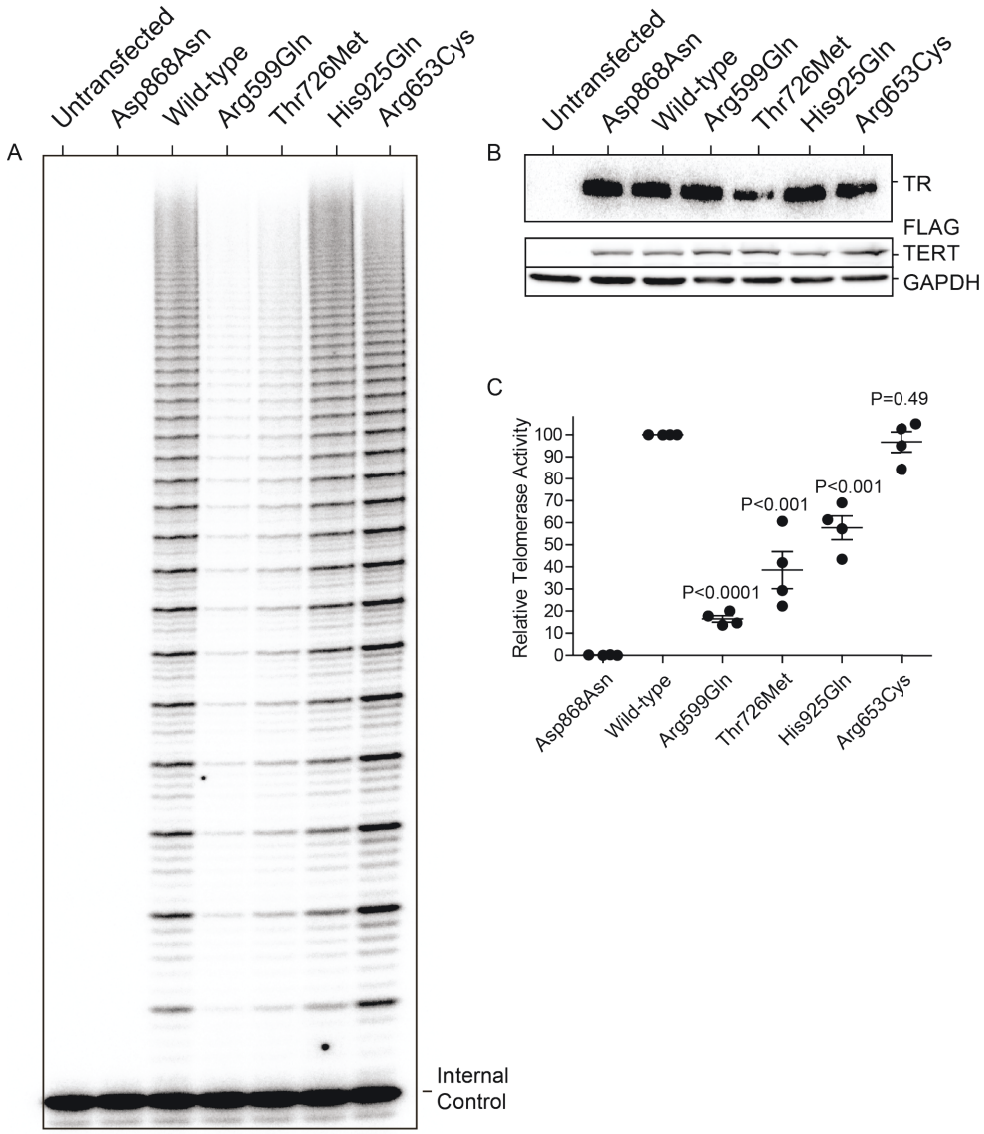
Supplementary Table 3. Minor allele frequency of *TERT* Variants found in COPDGene and LHS subjects*

Variant**	Previous report	COPDGene Controls (n=206)	COPDGene Cases (n=209)	Lung Health Study (n=83)
g.1253918C>T p.Pro1108Pro	rs35033501	0.019	0.029	0.108
g.1254594C>T p.Ala1062Thr	rs35719940	0.014	0.022	0.036
g.1255454G>A p.Val1035Val	rs181612536	0.005	0.005	0
g.1255520G>A p.His1013His	rs33954691	0.104	0.100	0.181
g.1260708G>T p.Arg951Arg	-	0	0.003	0
g.1264587G>A p.His925His	rs34528119	0	0	0.012
g.1264611C>T p.Thr917Thr	-	0	0.003	0
g.1268700C>T p.Thr839Thr	rs140124989	0	0.003	0
g.1271254G>A p.His816His	-	0	0.003	0
g.1278804C>T p.Val746Val	-	0	0	0.012
g.1278888,G,A p.Asp718Asp	-	0	0.003	0
g.1279430C>T p.Pro702Pro	rs151055240	0	0	0.012
g.1279439G>A p.Ala699Ala	rs33963617	0.012	0.005	0
g.1279505G>A p.Gly677Gly	rs33956095	0.012	0.012	0.036
g.1280387G>C p.Ala612Ala	rs34170122	0	0	0.012
g.1280411T>C p.Ala604Ala	rs33959226	0.007	0	0.012
g.1293767G>A p.His412Tyr	rs34094720	0.002	0.002	0.012
g.1294086C>T p.Ala305Ala	rs2736098	0.279	0.275	0.446
g.1294166C>T p.Ala279Thr	rs61748181	0.032	0.041	0.060
g.1295018C>T p.Arg29Arg	-	0	0	0.012
g.1295060G>A p.Arg15Arg	-	0	0	0.012

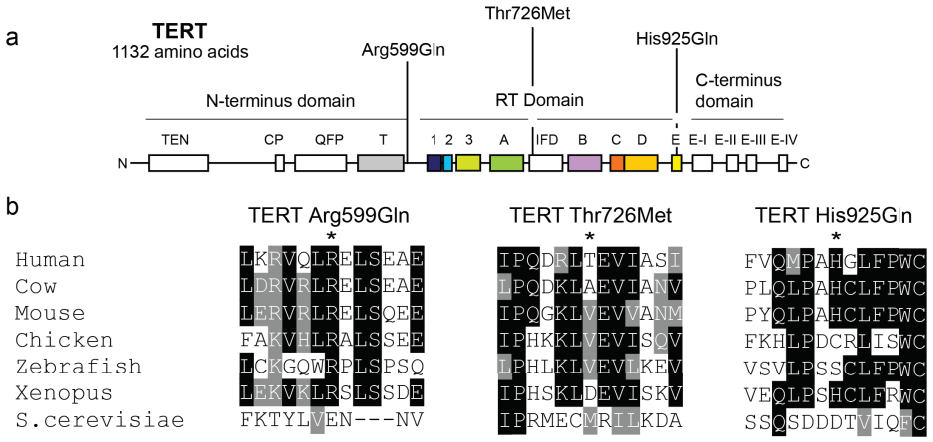
*No SNPs in *TR* were identified.
**Coordinates refer to hg19 version of the genome.



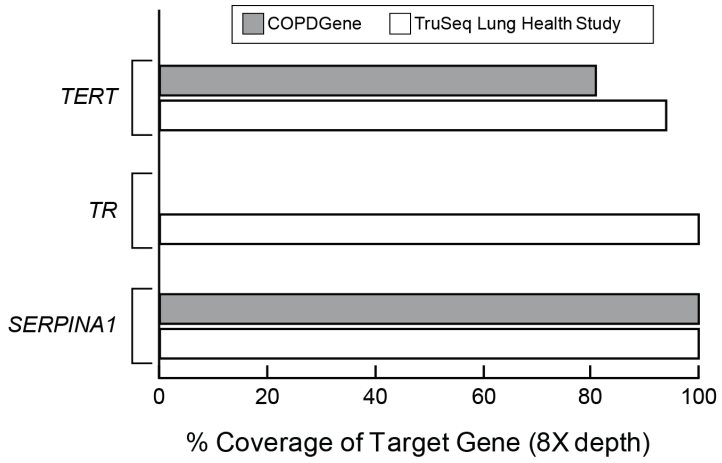
Supplementary Figure 1. Filtering strategy for identifying telomerase variants for functional analysis. Flow chart delineates a priori designed filtering strategy to identify rare telomerase variants in COPDGene and Lung Health Study (LHS). *Refers to publicly available VAAST software described in Yandell et al. *Genome Research* 2011. EVS refers to the Exome Variant Server, MAF to minor allele frequency, and VCF to virtual contact file.



Supplementary Figure 2. Functional consequences of telomerase variant identified in COPDGene controls. A. Gel image of telomeric DNA repeats generated from wild-type and variant telomerases reconstituted *in vivo* and immuno-purified. The total intensity of the DNA repeat products generated by TERT Arg653Cys shows similar activity as wild-type telomerase. A ^{32}P end-labeled 18mer oligonucleotide is included as an internal control for the recovery of DNA products. **B.** Northern blot for TR levels from immuno-purified telomerases and western blot for TERT expression in cells. Western blot performed with anti-FLAG and anti-GAPDH antibodies for ectopically expressed FLAG-tagged TERT and endogeneous GAPDH, respectively. **C.** Mean telomerase activity from four independent activity assays from two separate transfections. Enzymes were purified from cell lysates from two separate transfections and these experiments were done independent of those shown in Figure 1. Error bars represent standard error of the mean. P-values were calculated using Student's *t*-test and are two-sided.



Supplementary Figure 3. Conservation of mutated residues in TERT. A. Mutations fall in conserved catalytic domains of the telomerase reverse transcriptase. **B.** Evolutionary conservation of TERT variants across seven species. Mutated residues are labeled above the alignment. Alignments were generated with the online tools Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and BoxShade (http://www.ch.embnet.org/software/BOX_form.html).



Supplementary Figure 4. Depth of coverage in next-generation sequence data. Percent of sequences with 8X or greater coverage in the COPDGene (whole exome sequencing) and Lung Health Study cases (customized targeted panel). For the COPDGene coverage analysis, 11 exomes were randomly selected for this analysis using Binary Alignment/Map (BAM) files downloaded from the dbGaP portal. For the Lung Health Study, the coverage reflects the mean from 76 samples that passed quality control. The mean coverage calculation included exons and their flanking sequence. *SERPINA1* coverage reflects 20 nucleotides in exon 6 that contains the most common severe alpha-1 antitrypsin deficiency allele (PI*Z, rs28929474).

Chapter 3:
Exome sequencing identifies mutant *TINF2* in a family with pulmonary fibrosis

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Abstract

Short telomeres are a common defect in idiopathic pulmonary fibrosis, yet mutations in the telomerase genes account for only a subset of these cases. We identified a family with pulmonary fibrosis, idiopathic infertility and short telomeres. Exome sequencing of blood-derived DNA revealed two mutations in the telomere binding protein *TINF2*. The first was a 15 base pair deletion encompassing the exon 6 splice acceptor site, and the second was a missense mutation, Thr284Arg. Haplotype analysis indicated both variants fell on the same allele. However, lung-derived DNA showed predominantly the Thr284Arg allele indicating the deletion seen in the blood was acquired and may have a protective advantage since it diminished expression of the missense mutation. This mosaicism may represent functional reversion in telomere syndromes similar to what has been described for Fanconi anemia. No mutations were identified in over forty uncharacterized pulmonary fibrosis probands suggesting mutant *TINF2* accounts for a small subset of familial cases. However, similar to affected individuals in this family, we identified a history of male and female infertility preceding the onset of pulmonary fibrosis in 11% of telomerase mutation carriers with *TERT* and *TR* mutations (5 of 45). Our findings identify *TINF2* as a mutant telomere gene in familial pulmonary fibrosis, and suggest infertility may precede the presentation of pulmonary fibrosis in a small subset of adults with telomere syndromes.

The incidence of idiopathic pulmonary fibrosis (IPF) increases with age(1). IPF also has a strong genetic component as evidenced by the fact that as many as one-fifth of affected individuals report another family member with idiopathic interstitial fibrosis(2). Familial pulmonary fibrosis (FPF) is most frequently inherited as an autosomal dominant trait with age-dependent penetrance, and mutations in the telomerase genes are its most commonly identifiable cause(1). Loss-of-function mutations in the core telomerase genes, *TERT* and *TR,* can be identified in 8-18% of probands with FPF(1). More rarely, FPF displays X-linked inheritance(3), and mutations in dyskerin, the X-linked telomerase holoenzyme component encoded by *DKC1*, have been linked to sporadic(4) and FPF(5), and account for ~1% of cases (unpublished). Despite these recent discoveries, extra-pulmonary features of a telomere syndrome, such as liver disease and bone marrow failure, are often detected in pulmonary fibrosis patients who have short telomeres but in the absence of telomerase mutations suggesting the full complement of telomere genes in IPF is yet-to-be characterized(6, 7).

Lung disease is the most common manifestation of germline defects in telomere maintenance in adults(1, 8). In more severe forms, telomere-mediated disease manifests in children in the disorder dyskeratosis congenita, a mucocutaneous condition classically defined by abnormal findings in the skin, nails and the oral mucosa, and marked by a predisposition to bone marrow failure(9). In children with dyskeratosis congenita, mutations in *DKC1*, and in the telomere repeat interacting nuclear factor 2, *TINF2,* are the most common identified genetic causes(9). Here, we report identifying by exome sequencing a mutation in *TINF2* in an adult proband with FPF.

Materials and Methods

Human Subjects. Subjects were recruited to a Johns Hopkins study aimed at understanding the genetics and natural history of telomere-mediated disease(10, 11). The study was approved by the Johns Hopkins Medicine Institutional Review Board, and all the participants gave written informed consent.

Exome Sequencing. Exome sequencing was performed using the SureSelect XT All Exome V4 kit and sequenced on the Illumina HiSeq 2000 platform as described(4). Variants were called using the Genome Analysis Tool Kit (GATK) and annotated using ANNOVAR (http://www.openbioinformatics.org/annovar/)(4). Unique variants in telomere genes that were not found in the 1000 Genome Project Database and the Exome Variant Server were prioritized for additional studies.

Telomere length and DNA sequencing. Telomere length was measured on peripheral blood mononuclear cells by flow cytometry and fluorescence in situ hybridization(12). *TERT* and *TR* sequencing was performed as previously outlined(13). *TINF2* exon 6 was sequenced using the following primer sets: TINF2.E6F 5'-CCTGGAGACAATATGGTGTGG-3' and TINF2.E6R 5'-AGGCTGTTGATCCAATCCTG-3' (834 bp product). Because DNA derived from formalin-fixed paraffin-embedded tissues is fragmented, we used two primer sets to genotype the two variants: TINF2.E61F 5'-AGACCTTTTGAGGCAGTCCA-3' and TINF2.E61R 5'-CCTTGAAGATGGTCCCTGAGGAAG-3' for Δ15 (247 bp product),

and TINF2.E6.2F 5'-CAGGGACCATCTTCAAGGAC-3' and TINF2.E6.2R 5'-TGGAGGCTGCTCTTGTGCCCATG-3' for the Thr284Arg missense (250 bp product). The proband had no deviations from the reference sequence at primer binding sites.

Haplotype and clonality studies. Genomic DNA was extracted from peripheral blood using a Puregene kit (Qiagen, Valencia, CA), and from paraffin-embedded lung tissue using standard protocols. Variants were confirmed by Sanger sequencing and the haplotype of adjacent variants was determined by TA cloning PCR products into a pCR4-TOPO vector according to the manufactures' protocol (Life Technologies) and sequencing individual clones.

TIN2 expression studies. We measured *TINF2* mRNA levels in lymphoblastoid lines generated from the proband as well as healthy controls as previously described(3) using the following quantitative real time PCR primers: TINF2.E3F: GATTTTGGAGGCACAGGAAA and TINF2.E5R: CTGCATCCAACTCAGCACAT. To test the *in vivo* stability of mutant TIN2 proteins, we cloned the *TINF2* genomic locus (inclusive of introns and exons) into a pCDNA5/FRT/TO vector (Life Technologies). An N-terminal Myc tag was introduced immediately after the start codon to facilitate detecting the exogenous TIN2 protein. Site-directed mutagenesis was used to introduce mutations and constructs were sequence verified. Constructs were then transfected into HeLa Flp-In™ T-REx™ system, alongside the pOG44 vector, to generate isogenic, tetracycline inducible lines according to the manufacturer's recommendations (Life Technologies). Cells were treated with 1 μg/mL doxycycline for one week prior to

protein extraction. Total protein was extracted after cells were lysed in RIPA buffer with protease inhibitors (Roche) and SDS-PAGE was performed using standard procedures followed by transfer to PVDF membranes. Anti-Myc (clone 4A6, Millipore) and Tubulin (ab6046, Abcam) antibodies were used for immunoblotting and all membranes were scanned using an Odyssey Infrared Scanner (LI-COR).

Results

Exome sequencing identifies *TINF2* mutations in a proband with FPF

The proband presented with IPF at 49 years and died at age 50 (Figure 1A-1B). She did not have premature graying, blood count abnormalities, or any mucocutaneous features of dyskeratosis congenita. Her brother died from IPF at age 44. Both the proband and her brother had a documented history of infertility that did not respond to reproductive assistance. The family history was notable for two maternal uncles who died from cryptogenic liver disease in their 30s (Figure 1A). Lymphocyte telomere length in the proband was at the first age-adjusted percentile, and granulocyte length was below the 10^{th} percentile (Figure 1C-1D). There were no mutations detected in *TERT* or *TR* by Sanger sequencing; however, exome sequence analysis identified two mutations in *TINF2*. The first was deletion IVS5-7–c.605-612 that spanned 15 base pairs across the intron 5-exon 6 boundary (hereon referred to as Δ15). This mutation deleted the splice acceptor site, and thus predicted a functionally null TIN2 protein (Figure 2A-2B). In addition, there was a heterozygous substitution in exon 6, c.851 C to G, which predicted a missense variant p.Thr284Arg (Figure 2A-2B). The latter mutation had been described in an adult with short stature, dental loss, bone marrow failure, and lung disease(14), and

fell within the *TINF2* exon 6 hotspot within which the vast majority of mutations reported in children with dyskeratosis congenita have been identified(15-17) (Figure 2A).

Haplotype studies suggest acquired mosaicism of the *TINF2* genotype in the blood

To determine the phase of the mutations, we amplified and cloned the *TINF2* region encompassing the variants, and quantified the proportion of each clone by Sanger sequencing. Among the clones sequenced from blood-derived DNA (n=56), the wild-type allele was in 34%. The Δ15 and Thr284Arg mutations were detected in *cis* in 45% of the clones. The remaining clones contained either the Δ15 (17%) or Thr284Arg (4%) alone. The clonal heterogeneity suggested two compatible possibilities. The first is that there was mosaicism in the blood. The second is that there was low grade polymerase-mediated template switching in the PCR amplification reaction that contributed to the allele heterogeneity we detected. We tested for template switching by mixing plasmids that carried the Δ15 and Thr284Arg separately, and found rare PCR products that contained only wild-type *TINF2* or both products in *cis*. Template switching may thus explain the rare single variant clones detected in the blood. To test for mosaicism, we sequenced genomic DNA extracted from lung biopsy tissue and found the Thr284Arg variant predominated (40% of clones, 8 of 20 screened). Only rarely, in 8% of clones (2 of 26), the deletion was detected. These data were consistent with blood-derived contamination since the vast majority of blood-derived DNA clones contained the deletion (61%, Figure 2C). The results indicated the Thr284Arg was the germline mutation and the Δ15 represented a secondary acquired variant in the blood.

Segregation studies support mutant *TINF2* segregates with IPF phenotype

To test for segregation in the family, we sequenced genomic DNA from the proband's father and sister who had no telomere syndrome features and normal telomere lengths and found both mutations were absent (Figure 1A, 1C-1D). These findings, along with the maternal history of liver disease, implicated the patient's mother as a likely obligate carrier of the Thr284Arg mutation (Figure 1A).

***TINF2* deletion disrupts protein expression**

We examined the functional consequences of the mutations on endogenous TIN2 expression by measuring the mRNA levels in cells derived from the proband and found a reduction by quantitative real time PCR (37% compared with 100±21%, n=4 controls). This was consistent with the Δ15-Thr284Arg mutation causing nonsense-mediated decay. To directly examine whether the Δ15 mutation affected the expression of the missense mutation, we cloned the *TINF2* genomic sequence under the regulation of a tetracycline inducible CMV promoter in HeLa cells (Figure 3A). We compared the protein stability of TIN2 encoded by wild-type, Thr284Arg, and Δ15-Thr284Arg. For additional comparison, we included TIN2 Arg282His, the *TINF2* mutation which is the most commonly identified in severe pediatric telomere syndrome cases(15). Both missense *TINF2* mutations produced proteins that had comparable levels to wild-type by immunoblot (Figure 3B). However, the Δ15-Thr284Arg mutation abolished the long isoform of TIN2, and produced lower levels of a degenerate product that was smaller than the TIN2 short isoform consistent with the splice junction mutation disrupting protein expression (Figure 3B).

TINF2 mutations are a rare cause of FPF

We had previously screened 73 FPF probands for *TINF2* mutations in exon 6(6). In this study, we screened 40 additional FPF probands and found no mutations. These data suggested that *TINF2* mutations are a rare cause of FPF and explain the genetic risk in approximately 1% of cases.

A history of infertility may precede the onset of IPF in telomere syndrome cases

In both the proband and her brother, infertility preceded the onset of IPF and we tested whether this pattern was recurrent in other telomere syndrome cases. We reviewed the history of 45 consecutively evaluated pulmonary fibrosis patients with telomerase mutations in our Johns Hopkins-based study and documented a history of referral for reproductive assistance in five other cases (11%, 3 male and 2 females, *TERT* n=3, *TR* n=2). In all these cases, the infertility evaluations preceded the onset of IPF. Among the total of the seven cases, including the two in this family, reproductive assistance led to a successful birth in three cases (43%, 2 male, 1 female).

Discussion

Here we report mutations in *TINF2* in association with FPF. Our observations add to a growing body of literature underscoring the intimate connection between telomere dysfunction and IPF etiology and risk. The proband and her affected brother with IPF had no features of classic dyskeratosis congenita and no bone marrow failure, the predominant phenotypes heretofore linked to *TINF2* mutations that usually manifest

before the age of 10(15-17). As such, our exome findings are notable and indicate the spectrum of disease associated with *TINF2* mutations may at times, albeit rarely, include adult-onset disease in the absence of hematologic abnormalities. With the addition of *TINF2*, there are six known FPF genes(4, 5, 13, 18-20). Four of these fall in the telomere pathway, and together they explain approximately one-fifth of the familial clustering of pulmonary fibrosis (Figure 3C).

Telomere length is the primary determinant of disease severity in telomere syndromes(9, 11). Severe telomere shortening usually manifests in children with aplastic anemia, while attenuated telomere defects are associated with adult-onset disease complicated by pulmonary fibrosis or emphysema(8, 9, 11). Pulmonary fibrosis has been reported in two isolated cases adult cases with classic dyskeratosis congenita and aplastic anemia in association with *TINF2* mutations(14, 21). These two examples further underscore the rare nature of the isolated FPF presentation in the family we report here. The determinants of disease severity beyond telomere length in *TINF2* mutation carriers are not known, but it may be that some mutations are hypomorphic such as in this case given the adult-onset clinical presentation, or that other genetic modifiers that are yet-to-be identified affect the severity of the telomere defect. It is also possible that the acquired deletion in the blood may have had a protective effect against a bone marrow failure phenotype in the proband.

The mosaicism we report here points to functional reversion as a likely acquired event and, to our knowledge, this has not been reported in the telomere syndromes. Somatic

reversion is known to occur in genome instability syndromes and has been described in dyskeratosis congenita patients with *TR* mutations(22). In these cases, homologous recombination mediates reversion to the wild-type allele in the blood(22). In our study; however, we found evidence suggesting an acquired clonal event in the blood. The mechanisms by which this occurred are unclear, but because the acquired deletion abolished the expression of the missense mutation, it may have given a growth advantage to the hematopoietic clone where it arose (Figure 3D). The selective growth advantage may have arisen because *TINF2* heterozygous null mutations are better tolerated than exon 6 point mutations *in vivo*. Functional correction has been previously documented in Fanconi anemia where acquired insertions and deletions in hematopoietic clones restore mutant protein function(23). Our report indicates this form of mosaicism may also occur in telomere disorders and this possibility should be considered in the genetic evaluation and counseling of individuals at risk.

Although the mechanisms by which *TINF2* mutations cause telomere shortening remain to be fully elucidated, several pieces of evidence point to a dominant negative genetic model. First, the human mutations cluster in a discrete hotspot. Moreover, *TINF2* mutations have been suggested to interfere with telomere elongation by telomerase in cell culture systems(24). The example we report here of an acquired deletion in the blood abolishing expression of the mutant TIN2 further supports this evidence. Congruent with this model, Tin2 heterozygous mice that carry an analogous TIN2 hotspot mutation acquire telomere shortening when bred successively for multiple generations(25). Altogether, these observations suggest that *TINF2* exon 6 missense mutations likely

interfere with normal telomere function and that their effect on telomere length is likely more deleterious than heterozygous null alleles. Additional human cell line experiments are needed to fully test this model. Although the reversion may have protected against the bone marrow failure phenotype in the proband, to date reversion has not been detected before the age of 40(22). It is therefore possible that in the missense mutation in this family represents a hypomorphic allele. The short telomere length in the proband is consistent with the possibility that the reversion may have occurred later in life, although telomere elongation may take up to two generations to be restored in wild-type offspring of telomerase mutation carriers(26, 27).

The presentation in this family highlights an association between short telomeres and decreased fertility in humans. Telomere shortening causes infertility and germ cell apoptosis in male mice(28, 29), and has more recently been linked to decreased fertility in female mice(30). Our retrospective observations in telomerase mutation carriers with pulmonary fibrosis suggest infertility, both male and female, may be a first telomere syndrome presentation in some cases. As in the index family here, the infertility phenotype may be more pronounced in later generations because of genetic anticipation, the known pattern of earlier, more severe disease seen in autosomal dominant families with telomere syndromes(31, 32). This association between telomere shortening and infertility has implications for the care of telomere syndrome patients as affected patients may need earlier referral for reproductive assistance. A family history of infertility in association with pulmonary fibrosis may also raise the suspicion for an inherited telomere

syndrome. The telomere syndrome diagnosis should in turn be considered in the workup of unexplained infertility especially in the context of other telomere syndrome features.

The genetic diagnosis of telomere-mediated pulmonary fibrosis has important implications for treatment. In the lung transplant setting, pulmonary fibrosis patients are prone to otherwise rare complications caused by immunosuppressive medications that include excessive transfusion support for cytopenias, renal failure, and gastrointestinal bleeding(33). The link between *TINF2* and FPF therefore has implications for genetic evaluation as well as patient care.

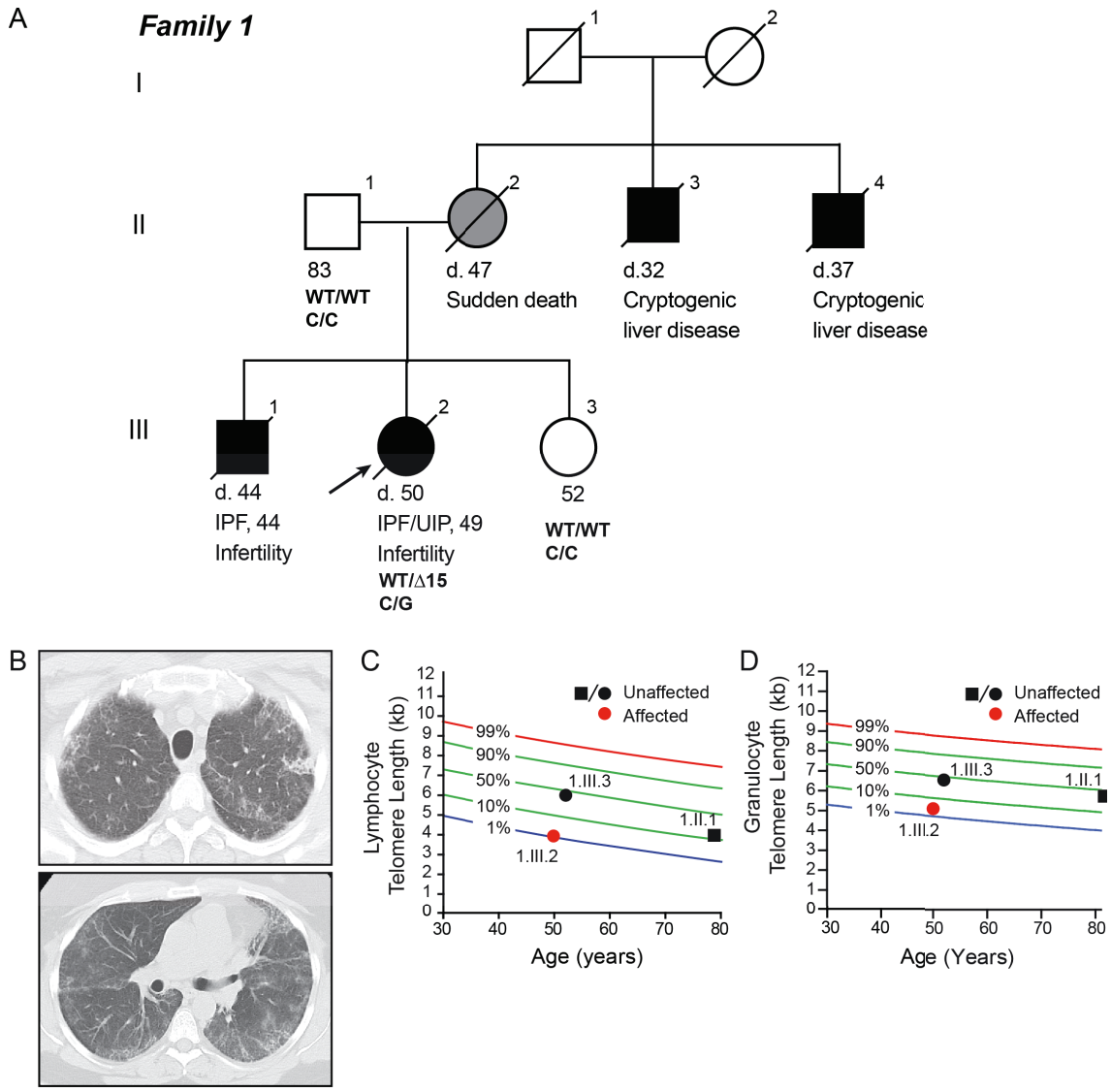

Figure 1. Clinical features of family with pulmonary fibrosis. A. Pedigree of index case with summary of the clinical history designated below. The proband is delineated by the arrow. Male family members are squares and females in circles. The black shade refers to individuals with clear telomere phenotypes and gray to probable obligate carrier of a *TINF2* mutation. *TINF2* genotypes are denoted adjacent to the individuals sequenced. WT refers to the wild-type reference sequence, and Δ15 refers to the deletion at the intron 5-exon 6 junction. The C/G genotype refers to the missense mutation. **B.** Lung window images from a chest CT scan from the proband showing peripheral honeycombing predominantly in the lung bases typical of IPF. The usual interstitial pneumonia (UIP) histology was subsequently confirmed on lung biopsy. **C.** Lymphocyte telomere length of affected and unaffected individuals from **(A)** are plotted relative to age-matched controls. **D.** Granulocyte telomere length relative to age-matched controls. Nomograms and percentiles were based on data from 192 controls. The pedigree identifiers refer to individuals in **(A)**.

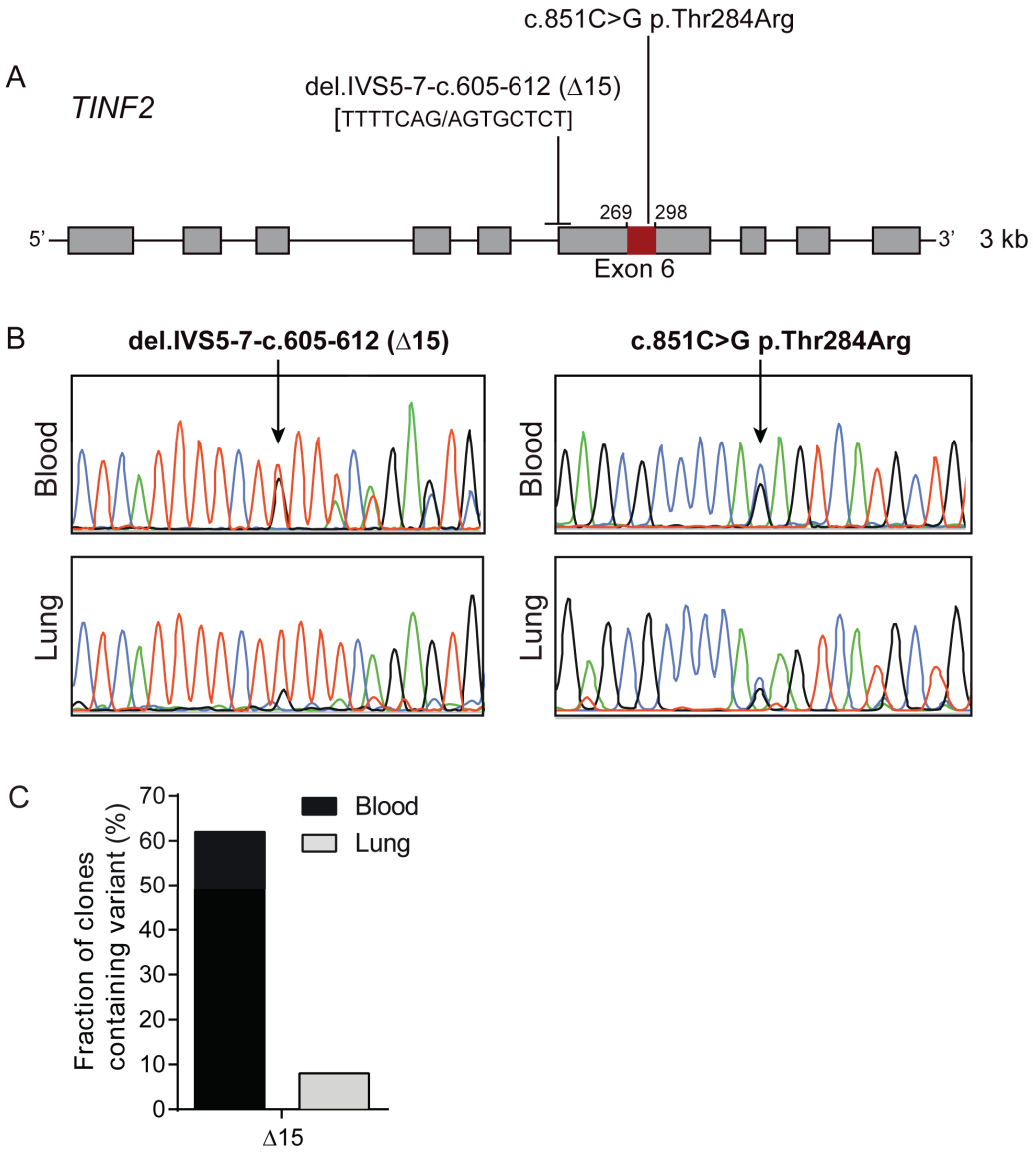

Figure 2. *TINF2* mutations studies in a proband with pulmonary fibrosis. A. Schema of *TINF2* genomic locus showing position of mutations detected by exome sequencing above. The exon 6 hotspot is highlighted in red. **B.** Chromatogram traces of sequence data from the proband derived from blood and lung genomic DNA. **C.** Percentage of each of the PCR-amplified products derived from clonal analysis in the blood as well as the lung.

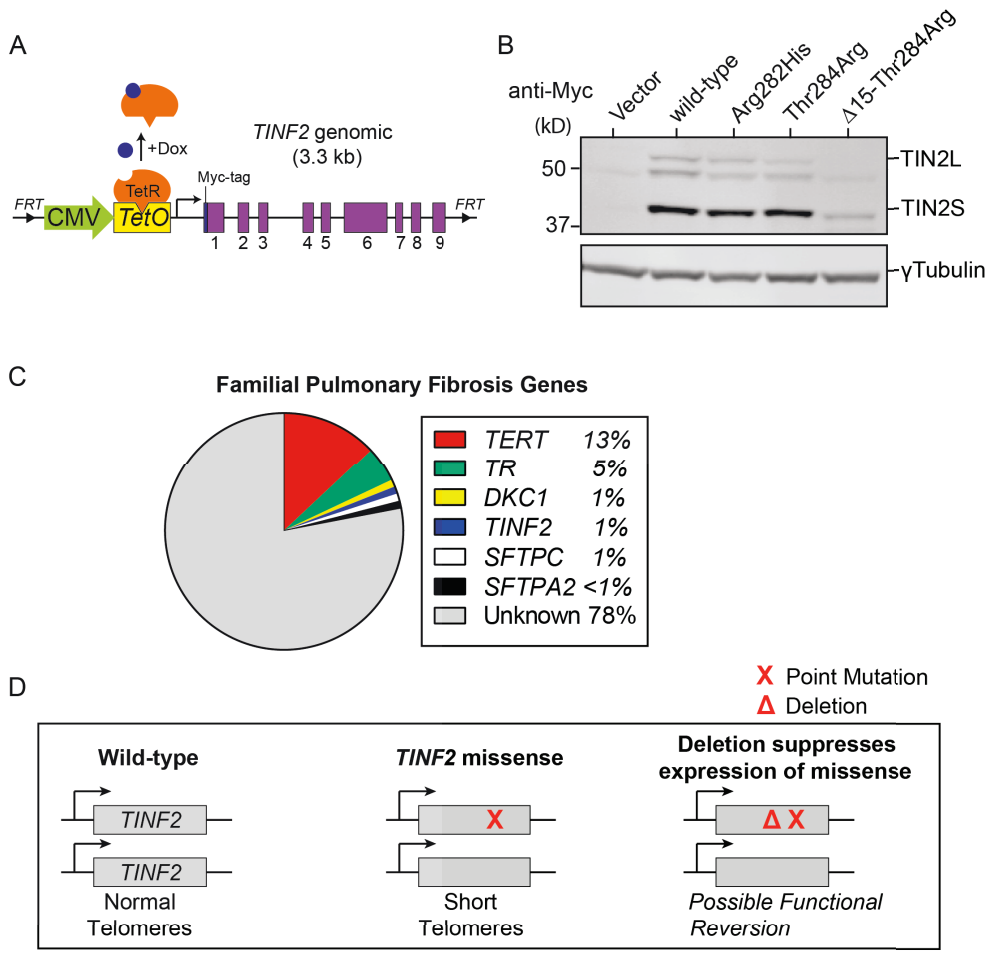

Figure 3. Consequences of mutations on TIN2 protein stability and proposed model of functional consequences of *TINF2* mutations. **A.** Schema of *TINF2* at its genomic locus inserted in *FRT* sites in HeLa cells. **B.** Immunoblot of Myc-tagged TIN2 expressed from a single promoter shows its two isoforms, TIN2L (long form) and TIN2S (short form) as described in Kaminker and Campisi *Cell Cycle* 2009. A third band, possibly representing a third TIN2 isoform, is also seen at an intermediate size between TIN2L and TIN2S. In contrast to the wild-type, and the two missense variants, Δ15-Thr284Arg abolishes the expression of TIN2L and creates low levels of a smaller degenerate protein product below the expected size for TIN2S. **C.** Pie chart illustrates the six known familial pulmonary fibrosis genes and their frequency estimates. **D.** Schema of model summarizing the putative functional consequences of the *TINF2* mutations within the context of genetic and functional studies delineated.

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Chapter 4:
Radiosensitivity and radionecrosis in the short telomere syndromes

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We report radionecrosis as a first manifestation of a germline mutation in telomerase. A 60 year old woman presented with defective wound healing after multiple surgical explorations of the chest wall to exclude breast cancer recurrence. At age 42 on routine mammography, she was diagnosed with stage I estrogen receptor positive left breast infiltrating ductal carcinoma. Lumpectomy with axillary node dissection revealed T1N0 disease. Adjuvant radiation therapy consisted of 50.4 Gy in 28 fractions followed by a 10 Gy boost in five fractions to the tumor bed. She did well for twelve years when a left rib fracture was incidentally noted on imaging; this prompted multiple needle biopsies which showed atypia but no malignancy. Open surgical biopsy revealed the anterior ribs within the radiation field were replaced with soft tissue, but no malignancy was found. The post-operative course was complicated by multiple wound infections requiring surgical debridement and failed healing of skin grafts that left her with an open chest wall wound (Figure 1A). She was evaluated at our center for a second opinion.

CT imaging showed fractured left ribs and chest wall abnormalities consistent with radionecrosis. In addition, in the contralateral lung, there was basilar honeycombing characteristic of idiopathic pulmonary fibrosis (IPF) (Figure 1A-1B). The family history revealed two siblings who died from IPF and myelodysplastic syndrome, a complex pathognomonic for the short telomere syndrome diagnosis(1). Genetic evaluation revealed a deleterious mutation in the telomerase RNA gene, *TR*, that was also found in her siblings (n.80, T>A). The mutation predicted disrupting TR's essential pseudoknot domain, and was associated with short telomere length, below the age-adjusted 10th

percentile (Figure 1C). Additional chest wall reconstruction was deferred given the known risk of respiratory failure after elective surgery and anesthesia in IPF patients(2). In the subsequent year, her wound improved with conservative care, but she developed worsening respiratory symptoms, and subsequently died cancer-free from end-stage lung disease.

In order to test whether short telomeres may predispose to radiosensitivity, we utilized a modified cell survival assay that is diagnostic of double strand break syndromes such as ataxia-telengiectasia (A-T)(3, 4). We examined lymphoblastoid cells derived from seven other telomerase and telomere gene mutation carriers (Figure 1C). Four of them were asymptomatic, two had IPF, and one had bone marrow failure. Cells from these subjects showed significantly compromised survival compared to controls and were similarly sensitive to A-T patients (Figure 1D-E). In 3 of 7 cases (43%), the surviving fraction at 1 Gy fell below at or below 21%, a threshold considered diagnostic of A-T in some settings(3). Neither the age nor the severity of the telomere defect predicted the 1 Gy surviving fraction in these subjects.

Radiosensitivity and radionecrosis are rare complications of treatment, but they are well-documented complications in patients with defective DNA double strand break repair such as A-T(5). The data we show here indicate patients with short telomere syndromes are similarly radiosensitive. To our knowledge, this is the first clinical case of radionecrosis in a telomerase mutation carrier. Sensitivity to radiation in the setting of telomere length abnormalities was first documented in telomerase null mice with short

telomeres(6), and our data establish the relevance of these observations to the clinical setting.

Syndromes marked by short telomeres manifest as IPF, emphysema and aplastic anemia(7). Mutations in telomere and telomerase genes are the most common cause of familial pulmonary fibrosis cases accounting for one-third of cases(7); they also rival alpha-1 antitrypsin deficiency as a risk factor for emphysema(8). Short telomere syndrome patients most often present in mid-late adulthood and have no associated dysmorphic features. A cancer diagnosis is estimated to affect up to 10% of severe cases, such as in dyskeratosis congenita, and predominantly manifests as myelodysplasia-acute myeloid leukemia and, more rarely, squamous cell head and neck carcinomas(9). There is a paucity of data regarding cancer treatment outcomes in this population. After hematopoietic stem cell transplantation, pulmonary fibrosis is a prevalent life-threatening complication, and lung shielding has evolved as a standard to minimize this risk(10). Awareness of this genetic diagnosis is important in clinical settings where ionizing radiation is utilized, such as in assessing the risk of X-ray-based imaging as a screening tool in pulmonary fibrosis, as well as in a preference for radiation-sparing regimens in hematopoietic stem cell transplantation. Suspected patients who have a suspicious personal or family history, especially of IPF-emphysema or myelodysplastic syndrome, should be identifiable by telomere length measurement combined with genetic testing.

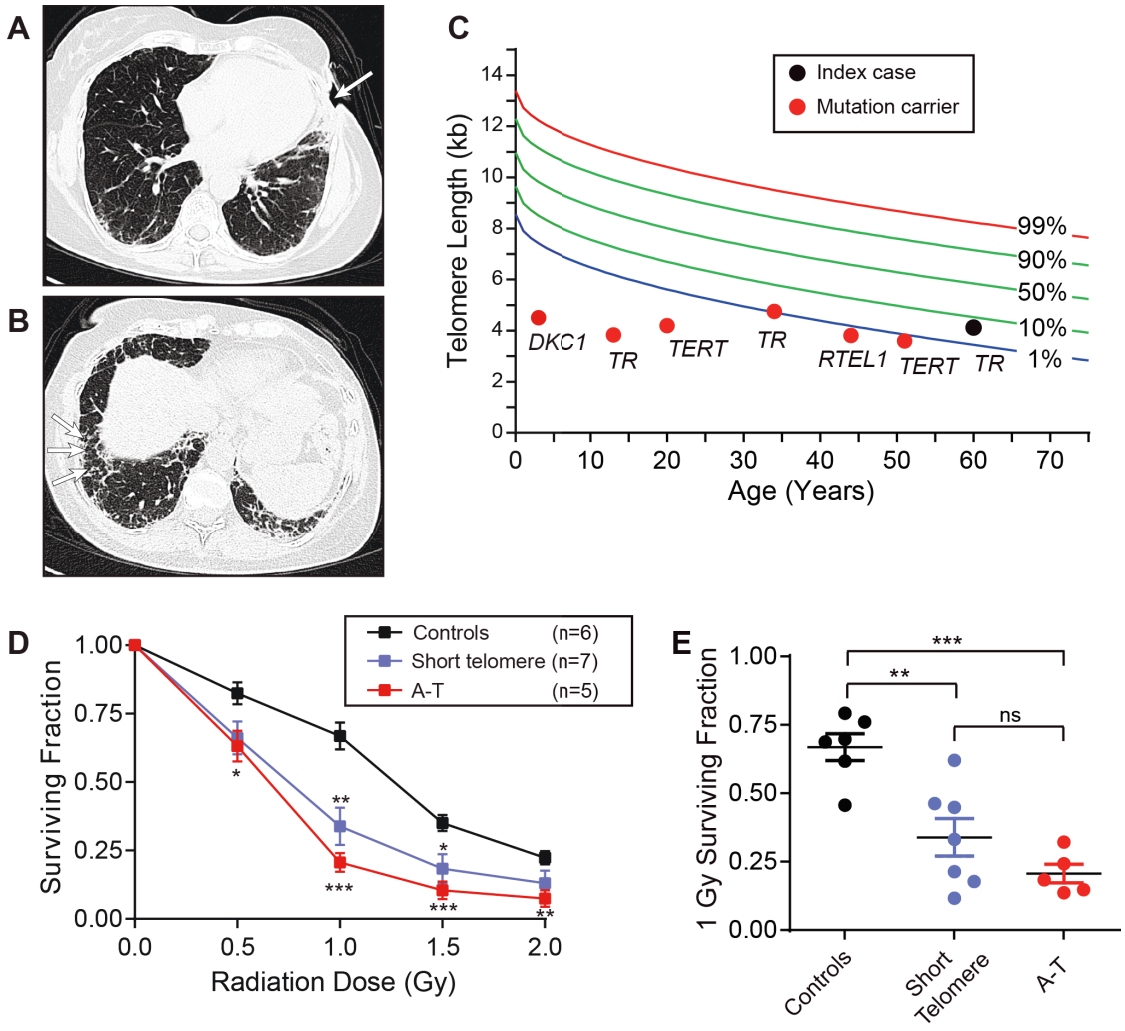

Figure 1. Clinical features and cell survival of cells with short telomeres after irradiation. A. Non-healing open left anterior chest wall wound (arrow) after open biopsy and failed grafts. **B.** Basilar honeycombing typical of idiopathic pulmonary fibrosis (arrows). **C.** Telogram shows telomere length of mutation carriers relative to age-matched controls (n=192). Telomeres were measured in peripheral blood lymphocytes by flow cytometry and fluorescence *in situ* hybridization. The labels indicate the mutant telomere gene in each of the cases. The red circles refer to individuals whose lymphoblastoid cell lines were studied *in vitro* (**D** and **E**). **D.** Dose response curve shows the surviving fraction of cells at each dose of ionizing radiation. Means reflect data from each group of controls, short telomere and ataxia telengiectasia (A-T) cells. Each cell line was assayed in quadruplicate, and the experiment was replicated twice. **E.** Individual data points for each of the cases at 1 Gy, a threshold which has been shown to useful in the diagnosis of DNA double strand break syndromes (Sun *et al.* 2002). Error bars refer to the standard error of the mean. *, ** and *** refer to two-sided P-values < 0.05, <0.01 and <0.001, respectively (Student's t-test, GraphPad Prism software). ns refers to a not statistically significant difference.

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Chapter 5:
Loss-of-function mutations in the RNA biogenesis factor *NAF1* predispose to pulmonary fibrosis-emphysema

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Abstract

Chronic obstructive pulmonary disease and pulmonary fibrosis have been hypothesized to represent premature aging processes. The two phenotypes at times cluster in families, but the genetic basis is not understood. Short telomeres have been shown to influence the development and progression of these phenotypes, yet among even telomere-mediated familial lung disease there is substantial locus heterogeneity. Compromised telomerase RNA (TR) levels are one molecular mechanism of disease and can be measured in patient cells. Using TR levels as a screening tool, we identified rare, frameshift variants in the gene nuclear assembly factor 1, *NAF1*, a box H/ACA RNA biogenesis factor, in pulmonary fibrosis-emphysema patients. Mutations segregated with short telomere length, low TR levels, and extrapulmonary manifestations including myelodysplastic syndrome and liver disease. A truncated NAF1 was detected in cells derived from patients, and in cells where the frameshift was introduced by genome editing, telomerase RNA levels were compromised. The mutant NAF1 lacked a conserved C-terminal motif, which we show is required for nuclear localization. Our studies identify a new disease gene in pulmonary fibrosis and emphysema, and outline the first report of haploinsufficiency for a box H/ACA RNP component in human disease.

Introduction

Lung disease is the third leading cause of death in the United States(1). Chronic obstructive pulmonary disease (COPD), which includes emphysema, along with pulmonary fibrosis (PF), are major culprits. Both diseases have been shown to co-cluster at times in families that show autosomal dominant inheritance; however, the genetic basis in the majority of these cases is not understood(2-5). A number of observations have linked the etiology of PF and emphysema to premature aging and to abnormalities in telomere length maintenance(3, 6-10). For example, abnormally short telomere length is a frequent finding in at least half of idiopathic PF patients(6, 11). Moreover, clinical features of a short telomere syndrome, including bone marrow failure/myelodysplastic syndrome, liver disease and infertility, are common in patients with sporadic and familial PF as well as in some patients with emphysema(6, 12-15). The diagnosis of a short telomere syndrome in these patients is relevant for patient care because the defect is systemic, and they show exquisite sensitivity to otherwise tolerated medications and procedures, especially in the lung transplantation setting(16). Mutations in the core telomerase enzyme components, *TERT*, the reverse transcriptase, and *TR,* the telomerase RNA component, were the first to be linked to inherited forms of PF; they are the most common identifiable genetic abnormality, and collectively they explain 15-20% of familial PF cases(2, 17-19). Smokers with telomerase mutations, especially women, are also prone to developing emphysema, alone or combined with PF(3). In smokers with severe, early-onset emphysema, *TERT* mutations were recently reported to be a risk factor in a proportion comparable to α-1 antitrypsin deficiency(3). However, in 70% of families with autosomal dominant pulmonary fibrosis, the genetic basis remains

uncharacterized(2), and, aside from *TERT*, the other heretofore identified telomere genes, *TR*, *DKC1*, *TINF2*, *RTEL1* and *PARN*, each explain only 1-3% of familial cases(13, 15, 20, 21).

Vertebrate TR shares with a family of non-coding RNAs a 3' box H/ACA motif; this domain is required for its stability and assembly into a mature telomerase holoenzyme complex(22-24). To overcome the locus heterogeneity seen in familial PF-emphysema, we took here the approach of classifying genetically uncharacterized cases by molecular abnormalities using TR levels as a stratification tool and examining candidate genes that have been implicated in TR biogenesis.

Results

Rare variants in NAF1 segregate with short telomere phenotype and low TR levels

To probe the genetic basis of uncharacterized familial PF-emphysema, we performed whole genome sequencing on five unrelated probands who had abnormally short telomeres, extra-pulmonary telomere syndrome features, and a family history consistent with autosomal dominant inheritance (fig. S1, A to C), but found no shared rare variants. To narrow the candidates, we sub-classified the cases by TR levels in lymphoblastoid cell lines (LCLs). Three probands had half the TR levels of controls as measured by quantitative reverse transcription real-time PCR (qRT-PCR) (fig. S1D), and we selected these for a candidate search that prioritized TR biogenesis genes. We included pontin (*RUVBL1*) and reptin (*RUVBL2*), ATPases that facilitate telomerase assembly(25), as well as *GAR1*, *SHQ1* and *NAF1,* which are involved in TR biogenesis(26-31). In JH1, a

proband with combined PF and emphysema, we found a heterozygous single base-pair insertion in exon 7 of *NAF1,* c.984insA, which predicted a premature stop after a short, out-of-frame amino acid sequence, S329Ifs*12 (Fig. 1A and B). This variant had not been seen in 9,006 individuals reported in public databases, and was absent in 134 controls we additionally sequenced (Fig. 1C). S329Ifs*12 was present in the proband's two siblings who had short telomere disease phenotypes, as well as in her mother who died from emphysema, but was absent in unaffected relatives (Fig. 1A and fig. S2). An affected sibling similarly had low TR levels relative to controls (Fig. 5D), as well as abnormally short telomeres, while non-carriers in the family had longer telomeres (Fig. 5E and F), albeit the telomeres were not as long in the proband's son, consistent with the known inheritance of short telomeres in offspring of telomerase mutation carriers(32-34).

To examine the prevalence of mutations, we sequenced *NAF1* in 25 additional genetically uncharacterized cases with PF, alone or combined with emphysema. Among them, we identified JH2, a woman with sporadic idiopathic PF and bone marrow failure, who carried a heterozygous two base-pair deletion, also in exon 7, c.956_957delAA, which predicted a frameshift followed by a premature stop, K319Rfs*21 (Fig. 1A and B). This second variant was also absent from the public databases and from the controls we sequenced (Fig. 1C). Leukocyte telomere length was abnormally short in this patient (Fig. 1E and F), and her peripheral blood cells failed several transformations with Epstein-Barr virus, presumably because of the severity of the telomere defect. Altogether, rare *NAF1* variants were detected in two of 30 (7%) of genetically uncharacterized PF-emphysema patients in our cohort.

A truncated NAF1 protein in mutation carriers

We examined the consequences of the frameshifts that we identified on protein stability. NAF1 is a 494 amino acid protein, and, if the truncated proteins were stable, a 339 and 338 amino acid product would be seen in JH1 and JH2, respectively (Fig. 2A). Protein lysates from patient-derived LCLs from JH1 and her sibling showed there was nearly half the level of full length NAF1 compared to unrelated controls and to the relative who did not carry the mutation. A lower molecular weight species that was only seen in mutation carriers was also detected; it corresponded to the predicted weight of the truncated protein (Fig. 2B). To determine whether the frameshift mutations could give rise to a shortened species, we expressed N-terminal Myc-tagged K319Rfs*21 and S329Ifs*12 (hereon K319fs and S329fs, respectively) under the control of a doxycycline-inducible CMV promoter at an isogenic locus in HeLa cells. In this system, we detected shortened NAF1 proteins that migrated at a molecular weight similar to the truncated species we observed in patient-derived cells, and to the predicted product for K319fs, respectively (Fig. 2C). When overexpressed, these mutants did not compromise TR levels (85% and 100% of control levels for K319fs and S329fs, respectively, P=0.7 for both), indicating they did not exert a dominant negative effect. In contrast, shRNA knockdown of the endogenous NAF1 decreased TR levels (Fig. 2D to F). The decrease in overall TR was independent of dyskerin because its levels were intact in LCLs from mutation carriers (Fig. 2B). These data suggested that NAF1 loss-of-function likely underlies the telomerase insufficiency in the patients we identified.

Disease-associated NAF1 alleles are functionally deleterious

To directly test the pathogenicity of the rare alleles, we compared their capacity to rescue TR after NAF1 knockdown in HeLa cells (Fig. 2G). K319fs failed to rescue TR, but S329fs was comparable to the wild-type allele in this system, suggesting it had residual function when overexpressed (Fig. 2H). To test the consequences of the S329fs allele on TR stability at its endogenous levels, we used CRISPR/Cas9 to knockin the mutation in pseudo-diploid HCT116 cells. A homozygous mutant clone, $NAF1^{S329fs/S329fs}$, was derived, and it expressed only the truncated NAF1 species (Fig. 2I and J). This clone exhibited significantly decreased TR levels (31% and 14% of control cells by qRT-PCR and northern blot, respectively, Fig 2K to I). Restoring full length NAF1 by lentiviral cDNA transduction partially rescued TR, consistent with a NAF1-dependent effect on telomerase levels in these cells (fig. S3, A to C).

NAF1 mutations impair nuclear localization

To understand how the mutant NAF1 proteins compromised TR, we examined the conserved domains within the truncated regions. K319fs and S329fs fell near a bipartite, lysine rich nuclear localization signal (NLS) that is functionally conserved in yeast(31, 35, 36) (Fig. 3A and fig. S4). K319fs disrupted the entire NLS, but S329fs interrupted only half the sequence (Fig. 3A). Additionally, K319fs, interrupted a highly conserved portion of the Gar1 homology domain (Fig. 3A and fig. S4). To test whether the truncated NAF1 proteins were defective for nuclear localization, we tracked the nuclear fraction of Myc-tagged proteins by immunufluorescence in the modified HeLa cells after treatment with leptomycin B, a blocker of nuclear export. Leptomycin B abolished the export of

NAF1^{WT} and it accumulated in the nucleus, as expected (fig. S5A). In contrast, when four lysines, previously linked to nuclear localization(36), were mutated to alanine (NAF1$^{4K>4A}$), there was a greater fraction in the cytoplasm (Fig. 3A to D). Similar to NAF1$^{4K>4A}$, NAF1^{K319fs} and NAF1^{S329fs}, were both enriched in the cytoplasm (Fig. 3C and D). Interestingly, the nuclear localization defect was more pronounced for NAF1^{K319fs}, consistent with our earlier results showing NAF1^{S329fs} is hypomorphic (Fig 3C and D, Fig. 2H). We also tested whether a nuclear localization defect was present in *NAF1*$^{S329fs/S329fs}$ HCT116 cells and documented a decreased NAF1 nuclear fraction by Western blot (mean 44% of controls, n=5 experiments, range 33-69%, P=0.002, representative gel in Fig. 3E). To assess the functional relevance of these observations, we probed whether an exogenous NLS can rescue the localization. We added an exogenous SV40-derived NLS sequence onto the N-terminus of each of the mutant proteins, and found that the nuclear localization was restored (Fig. 3A to D). However, for NAF1^{K319}, the exogenous NLS could not rescue TR levels after shRNA knockdown, suggesting the Gar1 homology domain altered in this mutant protein is also required for TR stability (fig. S5, B and C).

Discussion

We report here that *NAF1* loss-of-function mutations cause a short telomere syndrome that manifests as PF-emphysema as well as extrapulmonary telomere-mediated disease. Although mutations in the core telomerase components, *TERT* and *TR*, cause haploinsufficiency and autosomal dominant disease(17, 33, 37, 38), mutations in other telomerase biogenesis factors previously identified have shown recessive inheritance(39-

41). NAF1 may thus be unique among the RNA biogenesis factors with regard to its full dosage requirement for telomerase and telomere maintenance. Supporting this observation, a SNP near the *NAF1* locus is the only variant near a telomerase-related gene to be identified in genome wide association studies for telomere length, aside from *TERT* and *TR*(42, 43). rs7675998, which falls 40 kb downstream of *NAF1*, has an effect size on telomere length similar to SNPs near *TERT* and *TR*(42, 43). Our data suggest that this SNP affects telomere length by compromising NAF1 levels. Interestingly, rs7675998 has also been reported to be protective against melanoma(44). This likely also occurs by promoting telomere shortening, which is well-established to protect against melanoma risk(2). The exquisite sensitivity of telomere length maintenance to NAF1 levels that we identified here in Mendelian short telomere syndromes therefore sheds light on the molecular basis of disorders with complex inheritance.

The role of telomerase and telomere shortening in mediating lung disease has been studied in animal models. Although telomerase insufficiency alone does not cause lung pathology, telomerase null mice with short telomeres develop emphysema when exposed to cigarette smoke(8). The pathology has been linked to a lung intrinsic factors including cumulative injury caused by the genotoxic damage from cigarette smoke that is additive to existing telomere dysfunction in epithelial cells(8). When telomere dysfunction is induced in alveolar stem cells, they show hallmarks of senescence, and there is a lung remodeling that manifests in an emphysema-like phenotype that is associated with as recruitment of an inflammatory response resembling that seen in COPD(9). Our findings here, in the context of the published literature, highlight how telomere shortening is a

relevant mechanism for PF-emphysema susceptibility in a subset of patients beyond those with mutations in the telomerase core components. It is thus possible that efforts to reverse the telomere defect, through regenerative approaches, will influence the natural history of these progressive pathologies in the subset of patients with telomere-mediated lung disease.

Materials and Methods

Study Design. The goal of this study was to discover novel genes that cause familial short telomere syndromes. Probands with IPF-emphysema with pedigrees that show autosomal dominant inheritance were stratified by TR levels and genomic DNA was analyzed by whole genome sequencing and candidate gene examination. To test whether variants identified were disease-causing, their segregation with the short telomere phenotype and TR levels were examined, and their frequency in healthy controls was queried. To understand the functional consequences of mutations, a multi-species alignment was generated. The cellular and molecular consequences of the mutations were studied in patient-derived LCLs as well as in an inducible HeLa system where mutants were knocked in at an isogenic locus. CRISPR/Cas9 editing was used to introduce the mutations at the endogenous locus and study the effect on TR stability. All the immunofluorescence and northern blotting studies were performed blinded to genotype. All the studies were performed at a minimum of triplicate. Other details regarding the replication of the data are included in the respective figure legends.

Subjects. Subjects were recruited from 2007 to 2015 as part of the Johns Hopkins Telomere Syndrome Registry. Entry criteria included familial pulmonary fibrosis-emphysema or familial/sporadic cases with classic short telomere syndrome phenotypes and molecularly documented short telomere length as described(12, 45, 46). Control DNA (n=134) was obtained from the National Disease Research Interchange (NDRI) from individuals who had no lung disease, and the donors self-identified as being of European ancestry. Peripheral blood mononuclear cells (PBMCs) were transformed with

Epstein-Barr virus as described(47). Among the 25 genetically uncharacterized cases that were screened, sixteen had familial PF, and nine had sporadic PF-emphysema with extrapulmonary telomere syndrome features. The study was approved by the Johns Hopkins Medicine Institutional Review Board and all the subjects gave written, informed consent.

Telomere length measurement. Telomere length for all the studies shown was measured by flow cytometry and fluorescence in situ hybridization (flow-FISH) in a Johns Hopkins CLIA-certified facility as described(48). The flow-FISH method described includes extensive internal controls including MESF beads, cow thymocyte internal control, and standards included on each plate prepared using a robotic system as described(48). The facility's intra-assay coefficient of variation (CV) among the three replicate samples had a mean of 1.2% and 0.8% and the inter-assay CV was 1.8% and 0.7% for lymphocytes and granulocytes, respectively. Telomere data were plotted relative to a validated nomogram derived from healthy controls as previously described(18).

DNA extraction, next-generation sequencing and variant filtering. Genomic DNA from peripheral blood was isolated using the PureGene Blood Core Kit (Qiagen). For whole genome sequencing, genomic DNA libraries were prepared without amplification with the TruSeq DNA PCR-Free sample preparation kit (Illumina). Short read, paired end sequencing was carried out with the Illumina HiSeq2000 platform. Reads were aligned to the GRCh37 reference genome with the Illumina pipeline v2.0.2. Average depth of coverage was 45.1x and 98.3% of the genomes were covered at ≥20x.

VCF files were annotated using ANNOVAR, and the data were filtered based on their predicted effect on protein sequence (nonsense, frameshift, missense, and splice-altering), as well as their minor allele frequency (<0.0001) in each of the following publicly available sequencing databases: dbSNP build 129 (http://www.ncbi.nlm.nih.gov/SNP/), ESP6500 (http://evs.gs.washington.edu/EVS), and 1000Genomes (http://browser.1000Genomes.org). These databases were last accessed August 1, 2015.

NAF1 sequencing. Genomic DNA was derived from peripheral blood except for a sample which was from archived formalin-fixed paraffin embedded (FFPE) tissue and another from dried blood spots. To screen for *NAF1* mutations, we used a TruSeq Custom Amplicon Panel (Illumina) that included the coding *NAF1* sequence as well as flanking intronic sequence as described(3). In two cases, we analyzed the *NAF1* sequence from existing exome data that were generated and analyzed using previously described methods(15). Sanger sequencing was used to confirm variants and test for segregation in family members. The following primers were used for amplification and sequencing of *NAF1* exon 7: 5'-GGCTGATTACTGGCCTGTGTTAA-3' (forward) and 5'-CCTCCTGCTATGTAATGGCTCTAAA-3' (reverse) (476 bp). For FFPE tissues, primer sequences were modified to generate a smaller amplicon: 5'-GCATAATAGGCTTATTTTCTTCACC-3' (forward) and 5'-CAGAGAGAACCCAGATGTTTCCTTC-3' (reverse) (294 bp).

Telomerase RNA (TR) quantitative reverse transcription real-time PCR (qRT-PCR). Total RNA was extracted with the RNeasy Mini Kit (Qiagen) as recommended except DNAse digestion was performed using twice the volume and for an extended of 1 hour.

Random hexamer-primed cDNA was synthesized using the Superscript III Reverse Transcriptase kit (Invitrogen). Quantitative PCR of hTR was carried out as described(49, 50), respectively. For each experiment, the side-by-side comparisons were made on simultaneously prepared cDNA.

Immunoblotting studies. Immunoblotting was performed as described(49). Briefly, cultured cells were lysed using radioimmunoprecipitation assay (RIPA) buffer supplemented with a protease inhibitor cocktail (Roche). Proteins were resolved with SDS-PAGE, and after transfer to a nitrocellulose membrane, were blocked in Odyssey blocking buffer (LI-COR). The following primary antibodies were used: human NAF1 (rabbit, ab157106, 1:1000; Abcam), Myc (mouse, clone 4A6, 1:1000; Millipore), human Dyskerin (rabbit, sc-48794, 1:250; Santa Cruz), and GFP (mouse, 7.1 and 13.1, 1:1000; Roche) with loading controls actin (mouse, ab8226, 1:2000; Abcam), tubulin (rabbit, ab6046, 1:5000; Abcam), PARP (rabbit, 9542S, 1:1000; Cell Signaling Technology), or GAPDH (mouse, mAbcam9498, 1:1000; Abcam). Secondary antibodies were conjugated to dyes IR680 or IR800 (donkey, 1:10,000; LI-COR), and blots were visualized using an Odyssey scanner (LI-COR). Cell fractionation was performed using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific).

Statistical analyses. Statistical comparisons were made using GraphPad Prism software and all P-values are two-sided. Student's *t*-test was used for comparison of means and P-values less than 0.05 were considered significant.

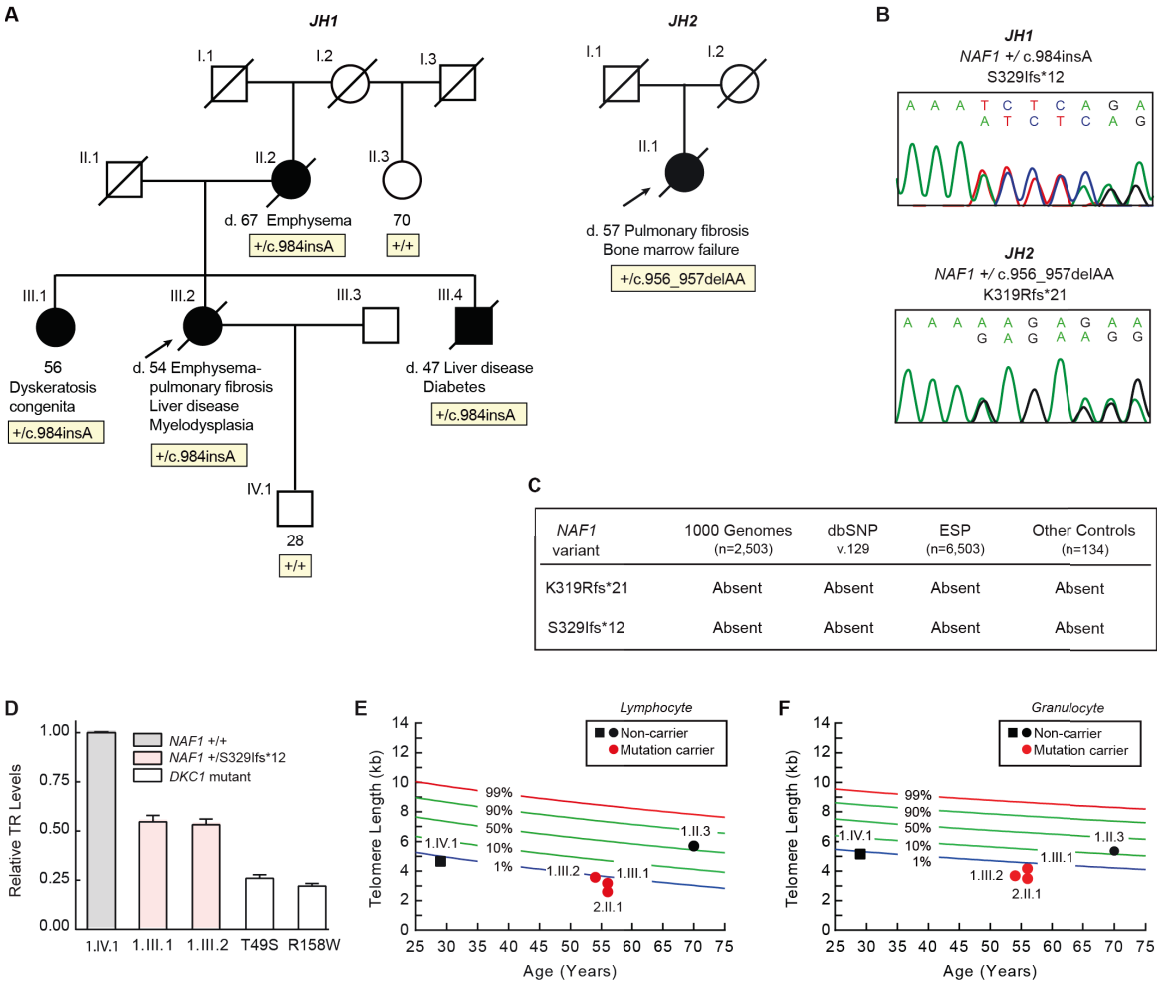

Figure 1. Rare *NAF1* variants segregate with short telomere disease phenotypes and low TR levels. A. Pedigrees of cases identified in a Johns Hopkins-based Registry (JH1, JH2) with *NAF1* genotypes shown below the individuals sequenced. **B.** Chromatograms of rare *NAF1* variants by Sanger sequencing. **C.** *NAF1* variants are absent in public database as well as additional controls we sequenced**. D.** TR levels by qRT-PCR in lymphoblastoid cell lines (LCLs) with pedigree identifiers referring to panel **A**. TR levels from *DKC1* mutation carriers serve as a positive control and the *DKC1* missense mutations are annotated below. Means are from 3 separate RNA isolations and experiments, and the error bars represent s.e.m. **E** and **F.** Age-adjusted telomere length in lymphocytes and granulocytes, respectively, measured by flow cytometry and fluorescence in situ hybridization (flowFISH) with pedigree identifiers corresponding to individuals in **panel A**. The nomogram was constructed from 192 healthy controls and the calculated percentiles are labeled.

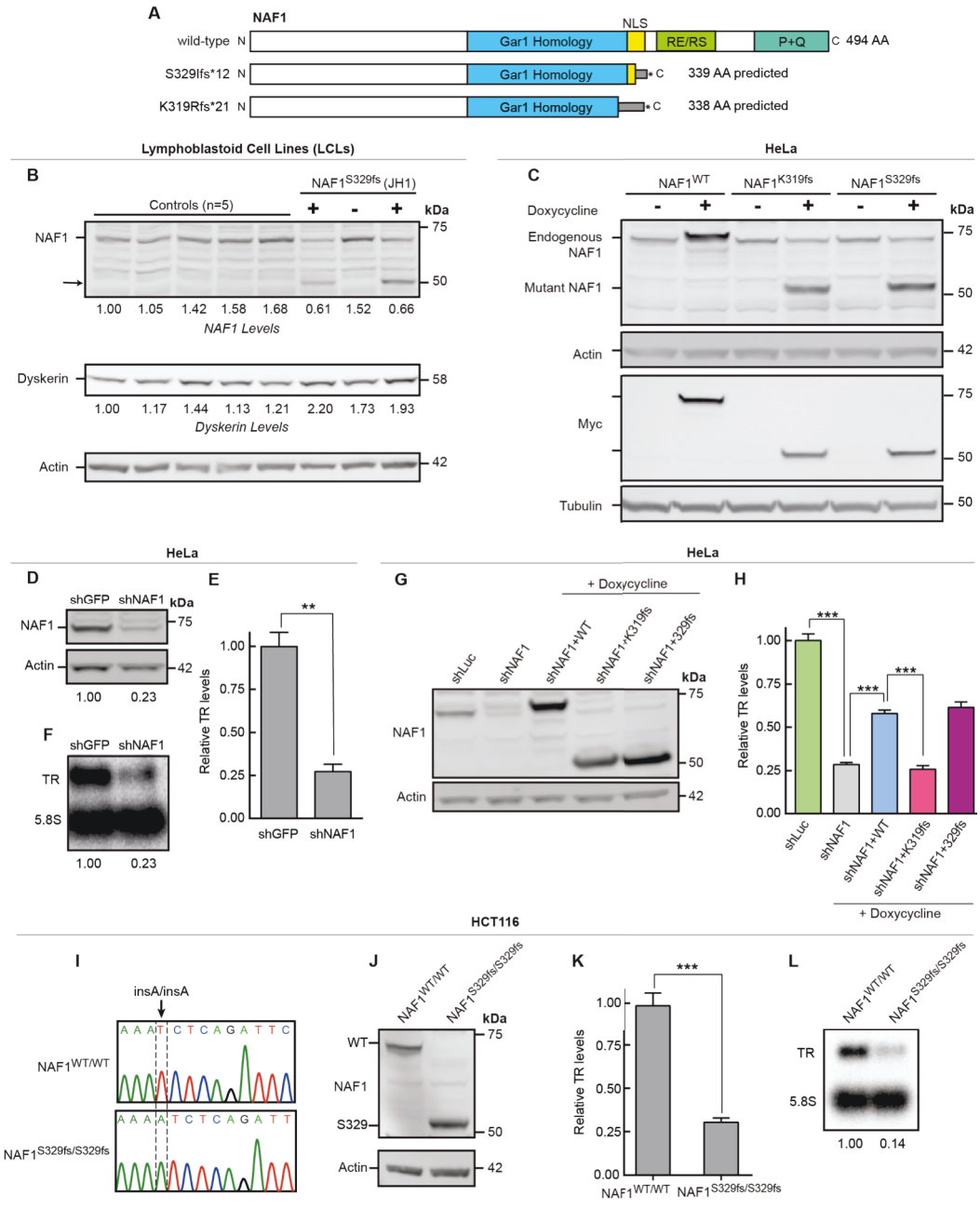

Figure 2. Disease-associated *NAF1* mutations disturb TR stability. A. Schema of NAF1 protein showing its conserved domains and predicted size of the truncated proteins (NP_612395.2). **B.** Western blot of NAF1 and dyskerin in lymphoblastoid cell lines derived from unrelated controls and individuals from JH1 family. The '+' refers to NAF1 S329fs heterozygous mutation carriers (1.III.1 and I.III.2) and '-' refers to a non-carrier family member (I.IV.1). Pedigree identifiers refer to **Fig. 1A**. **C.** Western blot for Myc-tagged NAF1 at an isogenic doxycycline inducible locus in HeLa cells. **D.** Immunoblot of NAF1 in HeLa cells after shRNA knockdown (day 8 timepoint). **E** and **F.** TR levels measured by qRT-PCR and northern blot,

respectively. Mean for qRT-PCR result represents 3 independent experiments (P=0.001). **G.** Immunoblot for NAF1 after stable shRNA knockdown in HeLa cells with doxycycline induction of Myc-tagged NAF1. **H.** TR levels measured by qRT-PCR after stable shRNA knockdown (48 hours post-induction). Mean shown is from 3 technical replicates. P-value for sLuc vs. shNAF is <0.0001, for shNAF1 vs. shNAF1+WT is 0.0002, and for shNAF1+WT vs. shNAF1+K319fs is 0.0003. **I.** Chromatogram of homozygous c.984insA S329fs disease-associated mutation knocked into the endogenous *NAF1* locus of HCT116 cells using CRISPR/Cas9. **J.** NAF1 immunoblot on lysates from CRISPR/Cas9 edited HCT116 cells. **K** and **L.** TR levels quantified by qRT-PCR and northern blot, respectively. For **K**, mean reflects 4 independent RNA isolations and P=0.0001. Error bars represent s.e.m. **indicates P<0.01 and ***P<0.001 (Student's *t*-test).

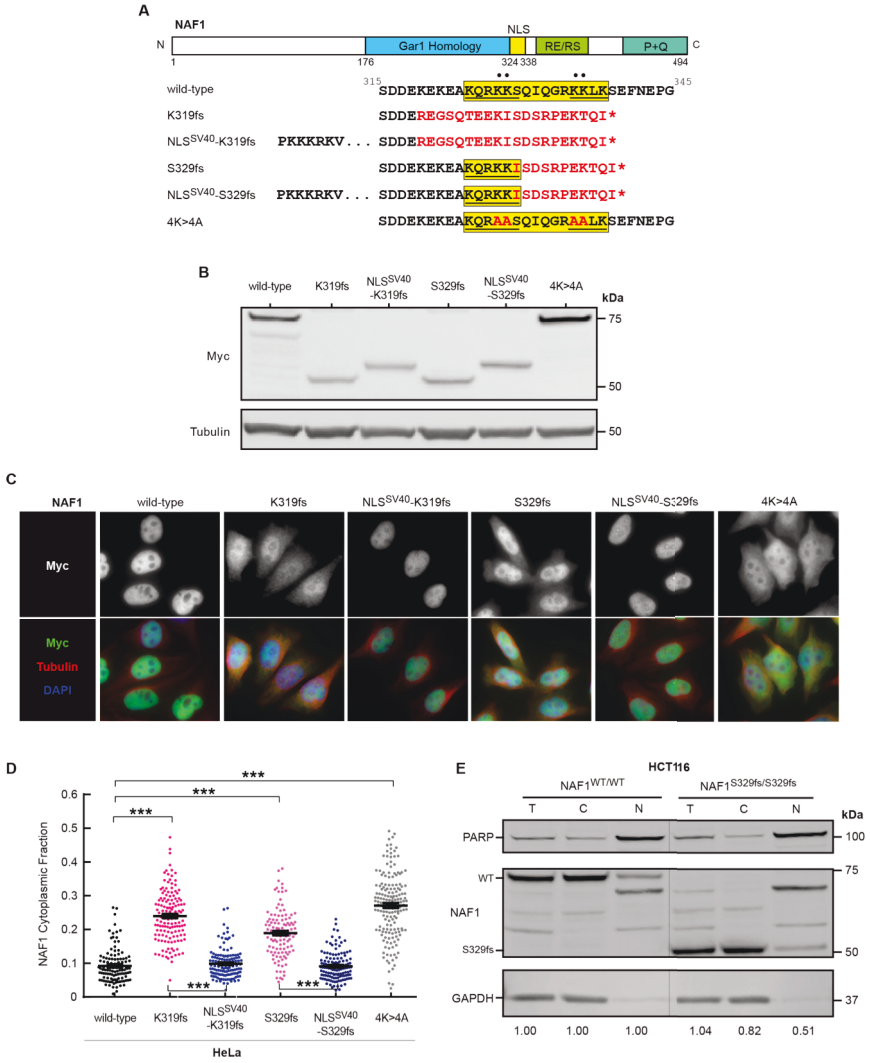

Figure 3. Mutant NAF1 lacks a C-terminal nuclear localization signal (NLS). A. NAF1 amino acid alignment within and flanking the NLS for disease-associated mutations and those studied for nuclear localization by immunufluorescence are additionally shown. The four conserved lysine residues are indicated by dots above, and the bipartite NLS sequences are underlined. **B.** Western blot of Myc-tagged NAF1 in the HeLa doxycycline-inducible system after 3 hour exposure to Leptomycin B (LMB). **C.** Immunufluorescence images showing sub-cellular localization of Myc-tagged NAF1 (green). Fluorophores in the three-color overlay are labeled to the left. Images were taken at 63X magnification (scale bar=20 microns). **D.** Quantification of the NAF1 cytoplasmic fraction represents the intensity of cytoplasmic anti-Myc staining (defined by tubulin positive area) relative to the nuclear staining (DAPI area). Approximately 100 cells were quantified for each cell line and mean values are shown with error bars representing s.e.m. Analysis was performed blinded to genotype. **E.** Western blot of total (T), cytoplasmic (C) and nuclear (N) protein lysates of parental HCT116 and CRISPR/Cas9 edited HCT116 cells. For the quantification shown below, the total and cytoplasmic fractions were normalized to GAPDH, while the nuclear fraction was normalized to PARP. ***P<0.001 (Student's *t*-test).

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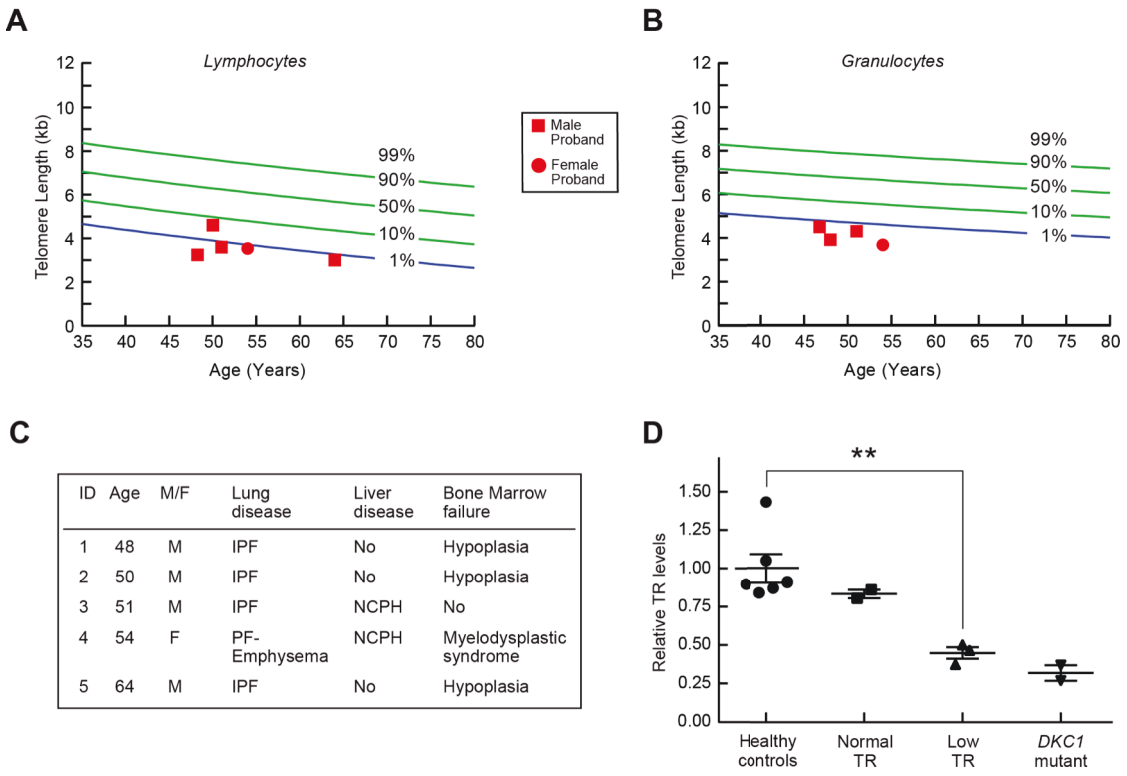
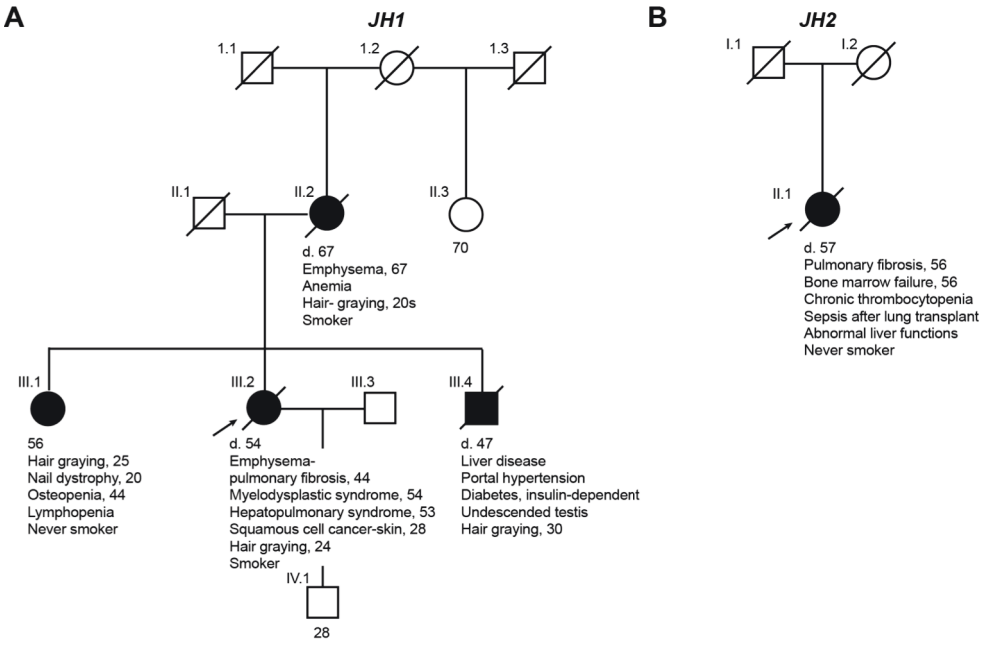
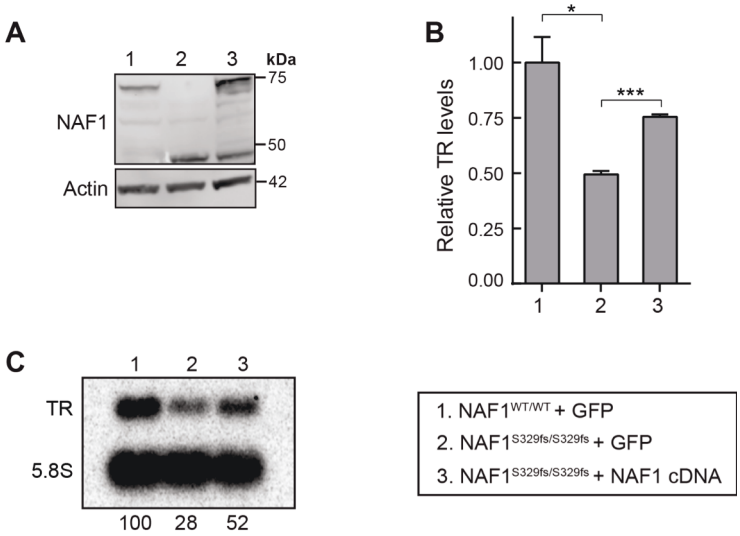


ID	Age	M/F	Lung disease	Liver disease	Bone Marrow failure
1	48	M	IPF	No	Hypoplasia
2	50	M	IPF	No	Hypoplasia
3	51	M	IPF	NCPH	No
4	54	F	PF-Emphysema	NCPH	Myelodysplastic syndrome
5	64	M	IPF	No	Hypoplasia

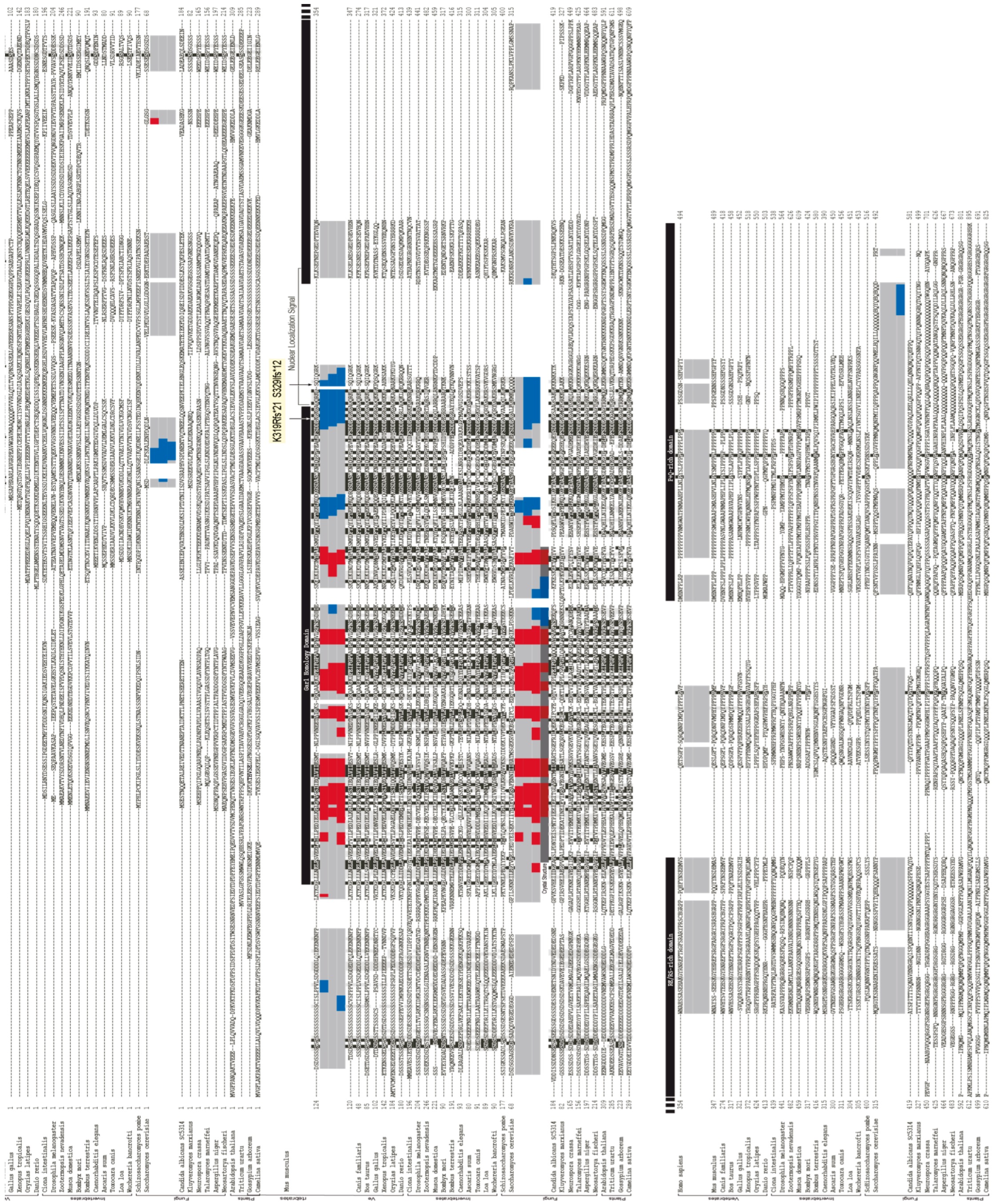
Supplementary Figure 1. Characteristics of pulmonary fibrosis-emphysema probands case studies. A and **B.** Age-adjusted telomere length in lymphocytes and granulocytes, respectively, of 5 genetically uncharacterized familial pulmonary fibrosis-emphysema probands with autosomal dominant telomere-mediated disease who were studied by whole genome sequencing. In one case there were insufficient events to measure granulocyte telomere length. Percentiles are shown based on data from 192 healthy controls. **C.** Clinical features of five probands studied by genome sequencing. Proband #4 is the index JH1 case. NCPH refers to non-cirrhotic portal hypertension. **D.** TR levels in the five probands measured by qRT-PCR in lymphoblastoid cell lines (LCLs) and stratified relative to controls (n=6). TR levels from *DKC1* mutation carriers (T49S and R158W) are included as a positive control. Means represent data from three independent experiments and RNA isolations. Data are expressed as mean ± s.e.m. and **P<0.01 (Student's *t*-test).



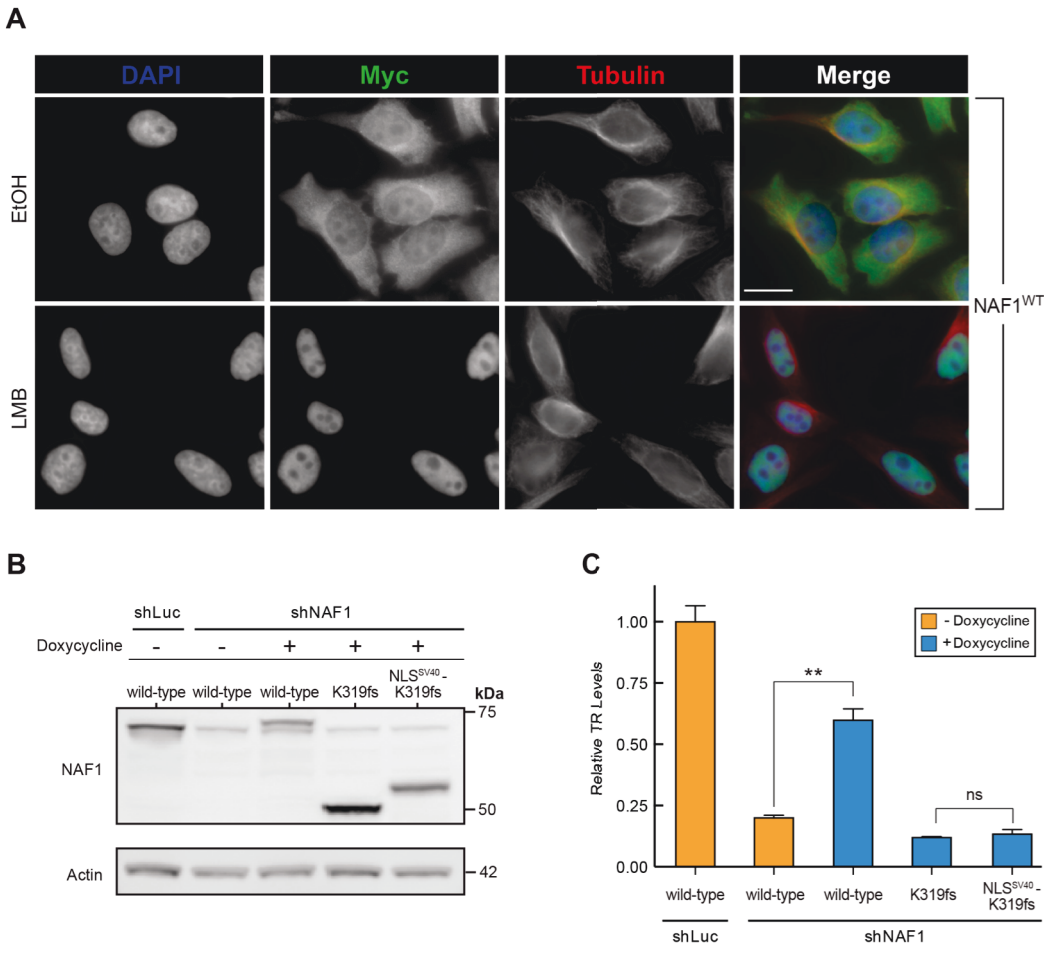
Supplementary Figure 2. Detailed clinical history of short telomere syndrome features in *NAF1* mutation carriers. The respective genotypes are shown in **Figure 1A.**



Supplementary Figure 3. Full-length NAF1 cDNA resuces TR levels in homozygous mutant HCT116 cells. A. NAF1 immunoblot on lysates derived from $NAF1^{S329fs/S329fs}$ cells that were transduced with GFP- or NAF1- containing lentiviral vectors. Parental HCT116 ($NAF1^{WT/WT}$) cells were also transduced with GFP as a control. **B.** TR levels measured by qRT-PCR with calculations based on 3 independent RNA isolations. **C.** Northern blot for TR with quantification shown below. TR levels were measured 10 days after lentiviral transduction. Data are expressed as mean ± s.e.m. *P<0.05 and ***P<0.001 (Student's *t*-test).



Supplementary Figure 4. Multi-species NAF1 alignment showing conserved motifs. Multi-species alignment of human NAF1 protein alongside representative vertebrate, invertebrate, fungal and plant species shows conserved sequences. The black shading indicates a minimum of 50% identity and grey shading 50% similarity. Conserved motifs and domains, including the nuclear localization signal (NLS), are denoted above the protein alignment. Alpha helices are colored in blue, beta-sheets are in red, and random coil in grey. The position of the two disease-associated frameshift mutations is denoted by arrows.



Supplementary Figure 5. Leptomycin B (LMB) blocks nuclear export and TR levels after nuclear localization is restored for NAF1^{K319fs}. **A.** LMB blocks export of NAF1 in HeLa cells. The grayscale panels show Myc-tagged wild-type NAF1 accumulates in the nucleus after a 3 hour exposure of LMB. Cytoplasmic and nuclear areas are defined by tubulin (red) and DAPI (blue) staining. Scale bar is 20 microns. **B.** Immunoblot for NAF1 in isogenic HeLa cells after shRNA knockdown and 48 hours after doxycycline induction. **C.** qRT-PCR of TR in the same experiment shown in **B** and means are from 3 technical replicates. All results shown here were replicated 3 times. Error bars represent s.e.m. and **P<0.01 (Student's *t*-test).

Curriculum Vitae
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Educational History

2009-present M.D./Ph.D. Candidate
Medical Scientist Training Program
Graduate Program: Cellular and Molecular Medicine
Thesis Adviser: Mary Armanios, M.D
Johns Hopkins University School of Medicine, Baltimore, MD

2005-2009 B.S., Biology with Honors and Distinction
Stanford University, Stanford, CA

Research Experience

2012-Present *The genetics and mechanisms of telomere-mediated lung disease.*
Doctoral Thesis, Adviser: Mary Armanios, M.D.,
Johns Hopkins University

2007-2009 *Live cell imaging of endocrine cell migration and differentiation during pancreatic islet development.*
Seung K. Kim, M.D., Ph.D., Stanford University

2006 *Role of hepatic steatosis in liver regeneration following partial hepatectomy.*
Nicholas Davidson, M.D., Washington University in St. Louis

2005 *Expression of the PPARγ ligand binding domain.*
Ellen Li, M.D., Ph.D., Washington University in St. Louis

Honors and Awards

2015 Michael A. Shanoff Research Award
Johns Hopkins University School of Medicine

2012 Graduate Qualifying Exam (with distinction)
Johns Hopkins University School of Medicine

2009 J.E. Wallace Sterling Award for Scholastic Achievement
Stanford University

Publications

Original Research

Stanley SE*, Gable DL*, Wagner CL, Carlile T, Hanumanthu VS, Khalil SK, DeZern AE, Applegate CD, Alder JK, Parry EM, Gilbert W, Armanios M. Loss-of-function mutations in the RNA biogenesis factor NAF1 predispose to pulmonary fibrosis-emphysema. *Science Translational Medicine*. 2016 Aug 10;8(351):351ra107.
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